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Faculdade de Farmácia

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Dissertation supervised by Professor José A. Belo and co-supervised by Professor Susana Solá

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Abstract

Diseases of the heart and circulatory system (CVD) are the leading cause of disability and mortality in developing countries, causing 3.9 million deaths year and accounting for 45% of all deaths in Europe, with expectations to increase further. Of all cardiac pathologies, coronary artery disease (CAD) has the highest mortality rates worldwide and is characterized by the accumulation of atherosclerotic plaques in the blood vessels of the heart leading to diminished blood flow. People who survive from CAD are permanently scarred, since the disease normally progresses to myocardial infarction, leading to a drastic decline in heart function, culminating in heart failure or even sudden death. Restoring function in an infarcted heart remains a challenge, mostly because cardiomyocytes (CM) have limited proliferative capacity and the supply of nutrients and oxygen to the cardiac cells is inefficient to allow the regeneration of the tissue. The only treatment available is heart transplantation, which, however, is not a sustainable solution due to organ supply paucity and immune rejection problems. Therefore, there is an urgency in identifying molecules and signalling pathways regulating coronary vessels morphogenesis, in order to translate them into regenerative solutions. Our group identified a collagen and calcium-binding EGF domain-1 (Ccbe1) protein associated with the formation of the ventricular coronary vessels.

Our current hypothesis is that the secretion of CCBE1 by the cardiac fibroblasts (CFs) will promote the development of new vascular networks from endocardial progenitors. Therefore, one of the goals of this thesis is to generate CFs from human induced pluripotent stem cells (hiPSCs), to posteriorly assess CCBE1 potential and role in the induction of angiogenesis in co-culture with endothelial cells. For that, we used a hiPS cell line previous established in our laboratory, and a protocol based on the modulation of the WNT pathway by small molecules. Using this protocol, we were able to obtain high quality progenitor cells, characterized by the expression of *ISLET1* and *NKX2.5*, as well as a proepicardial population marked by high expression levels of *WT1*, *TBX18* and *TCF21*, which is expressed in cardiac fibroblast lineage-specific progenitors. We also observed the expression of transcription factors associated with cardiac fibroblasts, *TCF21* and *HAND2*, as well as the staining of Vimentin, a protein specific of cardiac fibroblast.

In addition, since CCBE1 is thought to be involved in the ventricular coronary vessels formation and CFs possibly also take part in this process, we also evaluate the requirement of CCBE1 for the commitment of hiPSCs into CFs and understand if the modulation of this protein during the differentiation into CFs had any impact in the yield and/or morphology of these cells. We verified that the knockdown of *CCBE1* during the initial 6 days of differentiation negatively affects the expression of cardiac progenitor genes, as already had been observed in mouse cells, and more importantly, enhances the expression of proepicardial genes in the end of the proepicardial phase, indicating a crucial role for CCBE1 in regulating the fate choices between cardiomyocytes and cardiac fibroblasts

Keywords: angiogenesis, cardiac regeneration, hiPSCs, cardiac fibroblasts, CCBE1

Resumo

As doenças cardiovasculares constituem a principal causa de morte e morbilidade nos países desenvolvidos, causando 3.9 milhões de mortes por ano. De todas as patologias cardíacas, a aterosclerose tem a percentagem mais elevada de mortalidade globalmente, sendo caracterizada pela acumulação de ateromas nos vasos coronários levando à diminuição do fluxo sanguíneo. Os ateromas são constituídos maioritariamente por lipoproteínas que se infiltram nas paredes dos vasos, levando à proliferação das células cardíacas em redor, e culminando na disfunção das células endoteliais nessa área. Esta acumulação é um processo progressivo envolvendo vários interveniente e mecanismos, e em caso de rutura da placa forma-se um coagulo que causa a oclusão do vaso, resultando num enfarte do miocárdio (EM). Quando ocorre um EM existe uma privação de oxigénio e nutriente para o restante tecido cardíaco, levando à perda de cardiomiócitos. Para compensar a perda de tecido, o ambiente pro-inflamatório do coração estimula a ativação dos fibroblastos cardíacos em miofibroblastos, os quais excretam níveis levados de proteínas da matriz extracelular (ECM) levando à formação de tecido fibrótico (cicatriz). Inicialmente, a cicatriz desempenha um papel essencial mantendo a integridade estrutural do coração, prevenindo o colapso da parede ventricular. Contudo, como o tecido fibrótico não tem a mesma estrutura ou função que o músculo cardíaco, é incapaz de contrair ritmicamente ou de propagar corretamente os sinais elétricos pelo coração, levando ao aumento da rigidez da parede ventricular, agravando a condição cardíaca podendo mesmo causar falência cardíaca. Atualmente, o único tratamento para falência cardíaca consiste em transplantes cardíacos, um tratamento difícil devido à falta de dadores e possíveis complicações imunitárias, sem referir que nem todos os pacientes são elegíeis.

Tendo em conta a falta de tratamentos disponíveis para esta patologia, novas terapias regenerativas com base em células estaminais têm vindo a ganhar destaque. De facto, o estatuto pluripotente destas células torna-as capazes de se diferenciarem em vários tipos de células, consoante a estimulação fornecida. Assim, em caso de lesão, estas células podem originar células funcionais substituindo não só os cardiomiócitos lesados bem como os vasos coronários. É importante notar que para além de ser necessário repopular a área danificada com cardiomiócitos, também é indispensável promover a angiogénese para permitir o fornecimento de oxigénio e nutriente às células, aumentando a probabilidade e rapidez da regeneração celular. Em suma, promover o processo de angiogénese no tecido cardíaco danificado tornou-se num dos focos da terapia cardíaca,

sendo fundamental descobrir os mecanismos e células intervenientes, bem como a sua contribuição, na angiogénese cardíaca. Foi neste contexto que os fibroblastos cardíacos começaram a ganhar destaque. Inicialmente, os fibroblastos cardíacos só eram associados a condições patológicas, mas nos últimos anos tem sido demonstrado o seu contributo para a manutenção da homeostase cardíaca, com especial interesse para o seu potencial na angiogénese.

Deste modo, o principal objetivo desta dissertação foi a obtenção de fibroblastos cardíacos *in vitro* a partir de células estaminais, para poster análise do seu potencial e funcionamento durante a angiogénese em conjunto com células endoteliais. Para tal, através da modulação da via WNT através pequenas moléculas, utilizou-se um protocolo de diferenciação em fibroblastos cardíacos numa linha celular de hiPSCs já estabelecida no laboratório. Para avaliar a eficiência do protocolo de diferenciação vários ensaios foram utilizados, tais como ensaios de qPCR, para criar um padrão de expressão genética das células nas diferentes fases, recorrendo a múltiplos marcadores genéticos específicos para cada fase da diferenciação, e também ensaios de imunocitoquímica, para comprovar o fenótipo das células diferenciados e os dados previamente obtidos por qPCR Com base neste protocolo, os resultados de qPCR indicaram uma elevada expressão de *NKX2.5* e *ISLET1*, sugerindo a obtenção de uma população de progenitor cardíacos. Juntamente, experiências em citometria de fluxo demonstraram ao sexto dia de diferenciação, valores elevados de células marcadas positivamente para NKX2.5, confirmando a eficiência de diferenciação na primeira fase do protocolo. Relativamente, à segunda fase do protocolo de diferenciação, durante a indução de células do proepicardio, também se verificaram, através de qPCR, níveis elevados da expressão dos seus marcadores típicos *WT1* e *TBX18*, bem como para *TCF21* um marcador da transição epitelial-mesenquimatosa que é expresso em progenitores de fibroblastos cardíacos. Paralelamente, a expressão de WT1 também foi avaliada por imunocitoquímica, onde os resultados demonstraram a existência de uma população homogénea para a expressão deste marcador. Por último, a obtenção de fibroblastos cardíacos foi confirmada por qPCR, onde os marcadores *TCF21* e *HAND* apresentaram valores razoáveis, mas também por ensaios de imunocitoquímica, onde se verificou a presença de VIMENTINA.

Para além da obtenção fibroblastos cardíacos derivados de células pluripotentes, tivemos como objetivo avaliar o resultado da modulação do gene c*ollagen and calcium-binding EGF domain-1* (*CCBE1)* durante o processo de diferenciação, nomeadamente perceber

se a inibição deste gene poderia ter algum impacto na obtenção de fibroblastos cardíacos ou na morfologia dos mesmos. O gene *CCBE1* codifica para uma proteína da matriz extracelular envolvida na via de sinalização do factor de crescimento do endotélio vascular C (VEFG-C), um fator angiogénico produzido na forma inativa e processado proteoliticamente para a sua forma ativa através do complexo proteico constituído pelas proteínas CCBE1 e *A-disintegrin* e *metalloproteinase with trombospondin motifs 3* (ADAMTS3). Ou seja, CCBE1 é uma proteína essencial à síntese de VEGF-C. A maioria dos estudos realizados sobre o gene *CCBE1* são focados na síndrome Hennekam, em que as mutações no gene *CCBE1* levam a malformações do sistema linfático. Todavia, o gene *CCBE1* também tem vindo a ser estudado como um regulador chave da formação da vasculatura ventricular, a qual é dependente da sinalização por VEFG-C. De facto, alterações nesta via condicionam a formação de vasos coronários derivados do sinus-venoso. Assim, é possível que a proteína CCBE1, para além de contribuir para a formação dos vasos do sistema linfático, também regule o processo de angiogénese cardíaca. Para já, foi demonstrado o envolvimento da proteína CCBE1 durante a fase inicial do desenvolvimento cardíaco, influenciando a migração e a proliferação dos progenitores cardíacos. Por outro lado, o fenótipo causado pela disrupção do CCBE1 na fase inicial da cardiogénese é observável em pacientes com a síndrome de Hennekam.

Assim, tendo em conta o papel dos fibroblastos cardíacos (FCs) na angiogénese e a suspeita de que o gene *CCBE1* possa, também, estar envolvido neste processo, torna-se necessário investigar uma possível ligação molecular entre ambos. Em particular, perceber de que forma a modulação de *CCBE1* poderá afetar a formação de fibroblastos cardíacos. Os resultados obtidos neste trabalho demonstram que o gene *CCBE1* não é altamente expresso em FCs. Contudo, estes resultados não invalidam o facto de estas células poderem expressar apenas este gene após contacto com o VEFG-C. É possível que, quando estimuladas com VEFG-C imaturo, uma cascata de sinalizações celulares seja ativada nos FCs levando à produção de CCBE1. Esta, por sua vez, poderá processar o VEFG-C e disponibilizá-lo para a angiogénese cardíaca. Todavia, estudos futuros serão necessários para confirmar esta hipótese.

Não obstante, como a expressão do gene *CCBE1* é elevada durante a primeira fase de diferenciação, utilizando a mesma linha celular com a qual foi estabelecida o protocolo de diferenciação, inibiu-se o gene *CCBE1* através da adição de doxiciclina (DOX) às células. Após várias otimizações, conseguiu-se obter, ao sexto dia de diferenciação, uma

eficiência de inibição de 44% em células expostas à DOX, tendo os resultados confirmado que a inibição de *CCBE1* influencia a expressão de genes associados ao estado de progenitores cardíacos, tal como já demonstrado em células de ratinho. Contudo, resultados inesperados foram obtidos quando se caracterizaram as células de proepicardio tratadas com a DOX, as quais apresentavam, ao décimo segundo dia de diferenciação, níveis de expressão dos marcadores de proepicardio mais elevados, comparativamente às células controlo (sem DOX).

Em suma, neste trabalho foi possível não só estabelecer um protocolo de diferenciação em fibroblastos cardíacos, a partir de células pluripotentes, como também confirmar a importância do gene *CCBE1* em células humanas durante a fase de diferenciação em progenitores cardíacos. Além do mais, foi possível observar uma possível dualidade na modulação do gene *CCBE1* durante a fase inicial de diferenciação. Por um lado, a inibição de *CCBE1* parece condicionar a expressão dos genes típicos de progenitores cardíacos, por outro, parece promover a expressão de genes de proepicardio durante a fase de diferenciação em proepicardio, um resultado interessante que merece ser mais aprofundado.

Palavras-chave: fibroblastos cardíacos, regeneração, angiogénese, CCBE1

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Abbreviations

AAVS1 – adeno-associated virus integration site
ADAMTS3 - A-disintegrin and metalloproteinase with trombospondin motifs3
BS - blocking solution
CAD – coronary artery disease
CC - cardiac crescent
CCBE1- collagen and calcium-binding EGF domain 1
CDS – cardiosphere cells
CF – cardiac fibroblasts
CMs – cardiomyocytes
CPCs – cardiac progenitor cells
CRISPR – clustered regularly interspaced short palindromic repeats
CRISPRi – CRISPR interference
dCas9 – deactivated Cas9
DDR2 – discoidin domain collagen receptor 2
EBs – embryoid bodies
ECM – extracellular matrix
ECs – endothelial cells
EMT – epithelial-to-mesenchymal transition
EPDCs – epicardial-derived cells
EPDCs – epicardial-derived cells
ESCs – embryonic stem cells
ESCs – embryonic stem cells
FGF – fibroblast growth factor
Fsp1 – fibroblast-specific protein 1
G-CSF – granulocyte colony-stimulating factor
hiPSCs – human induced pluripotent stem cells
HLA – human leukocyte-antigen
ICC – Immunocytochemistry
ICM – central inner cell mass
iPSCs – induced pluripotent stem cells
KD – knockdown
KO – knockout
KRAB – krüpple associated box
LVEF – left ventricular ejection fraction
MI – myocardial infarction
MMPs – matrix metalloproteinases
MSCs – mesenchymal stem cells
PCR – polymerase chain reaction
PDGFR-α – platelet-derived growth factor receptor tyrosine kinase-alfa
PS – primitive streak
RT – qPCR - Real-time quantitative polymerase-chain reaction
SCs – stem cells
SHF – second heart field
TIMPs – tissue inhibitors of metalloproteinases
UCB – umbilical cord blood

VEGF – vascular endothelial growth factor

Units

% – Percentage
ºC – degree Celsius
mg – microgram
mL – milliliter
ng – nanogram
rpm – rotations per minute
μg/mL – microgram per milliliter
μL – microliter
μM – micromolar

Chapter 1 – General Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in developing countries, causing 3.9 million deaths per year and accounting for 45% of all deaths in Europe[1]. Furthermore, according to the 2016 statistics of the *World Health Organization*, within cardiovascular diseases, coronary artery disease (CAD) presents the highest mortality rates worldwide, a trend maintained during the last 15 years, despite the medical advances in prevention and treatment of this pathology[2,3]. CAD is a multi-factorial condition involving genetic predisposition, environment, as well as diet and lifestyle, which entails a panoply of factors that increase the occurrence of this pathology, such as smoking, alcohol intake, abdominal obesity, diabetes mellitus, low-density lipoprotein (LDL) cholesterol, high blood pressure (hypertension) and sedentary lifestyle[3–6]. Current diagnosis for CAD starts with noninvasive studies to determine myocardial function. If ischemic disease is suspected, coronary angiography is the current standard of care to evaluate the atherosclerotic disease. Treatment for CAD includes a combination of revascularization procedures and medical therapy[7]. Due to the high surgical risk of the revascularization procedures, therapies to reduce ischemic disease and regenerate the heart is an area of active research. In this context, biomedical research in cardiac regeneration and angiogenesis process recurring to stem cell-based strategies has been gathering especial attention to replace the lost cardiac tissue and/or repair the damage by promoting neovascularisation and endothelial repair, ultimately improving myocardial perfusion. Inclusively, some treatments are already in clinical trials phases, so it is expectable that in a few years, better and highly curative technologies became available to for restoring myocardial function in heart disease[8].

1.1 Coronary artery disease (CAD) pathology

Coronary artery disease is characterized by the accumulation of atherosclerotic plaques (atheroma) in the blood vessels of the heart, leading to diminished blood flow (Figure 1.1)[9]. Atherosclerosis is a progressive process that involves several intervenients and mechanisms such as the dysfunction of endothelial cells in the atherosclerotic area, rendering them unable to control the vascular constriction and causing an over-release of vasoconstrictors and a low release of vasodilators[3,10]. Likewise, the infiltration of cholesterol-containing lipoprotein particles and macrophages in the vessel's walls, which in turn incite smooth muscle cell proliferation, leads to the formation of an atheroma

which bulges into the lumen of the vessels, increasing the resistance to flow[3,10,11]. The cellular composition of the atheroma has an important role in defining its stability and clinical consequences. An atheroma core composed mostly of lipid-rich macrophages surrounded by a thin fibrous layer is more unstable than an atheroma that the core is mostly composed of smooth muscle cells with a thick fibrous covering layer, thus being more likely to rupture[4,6,12]**.** The rupture of plaques originates a blood clot that causes a total occlusion of the vessel resulting in myocardial infarction (MI) (Figure1.1)[12]. When the blood clot forms, it deprives the downstream muscle cells of receiving the appropriate oxygen and nutrient (ischemia), ultimately leading to tissue loss[3,10], specifically cardiomyocytes (CMs). This loss of CMs is counteracted by the formation of a fibrotic scar which in a short-term has a pivotal role in maintaining the structural integrity of the heart, preventing the collapse of the ventricular wall[9,13–15]**.** The formation of a fibrotic scar is a complex and dynamic process. First, the necrotic CMs death triggers an inflammatory response, resulting in the infiltration of macrophages and other immune cells in the injury area in order to remove the cell debris, enabling the beginning of the healing process[16]. Simultaneously, the inflammatory microenvironment, rich in cytokines and chemokines, promotes an activation of cardiac fibroblasts, which transdifferentiate into myofibroblasts[17]. These cells exhibit both smooth muscle cells and cardiac fibroblast cells characteristics, thus having a migratory and contracting phenotype and, most importantly, an extensive endoplasmic reticulum to allow the synthesis and secretion of large amounts of ECM proteins, mainly collagen[13]. As referred above, this initial deposition of ECM maintains the integrity of the heart, however the persistent activation of myofibroblast causes an unbalance between the ECM degradation and generation, resulting in excessive deposition of collagen in the ventricular wall, originating scar (fibrotic) tissue. Since the scar tissue does not have the same structure or functions as the native myocardium, it is unable to contract rhythmically or properly conduct the electric signals and increases the ventricular wall stiffness[13,18]. This leads to the development of arrhythmias and an overstress of the heart to compensate for the compromised cardiac output, overall affecting the cardiac performance, and potentially leading to heart failure[9,13,14].

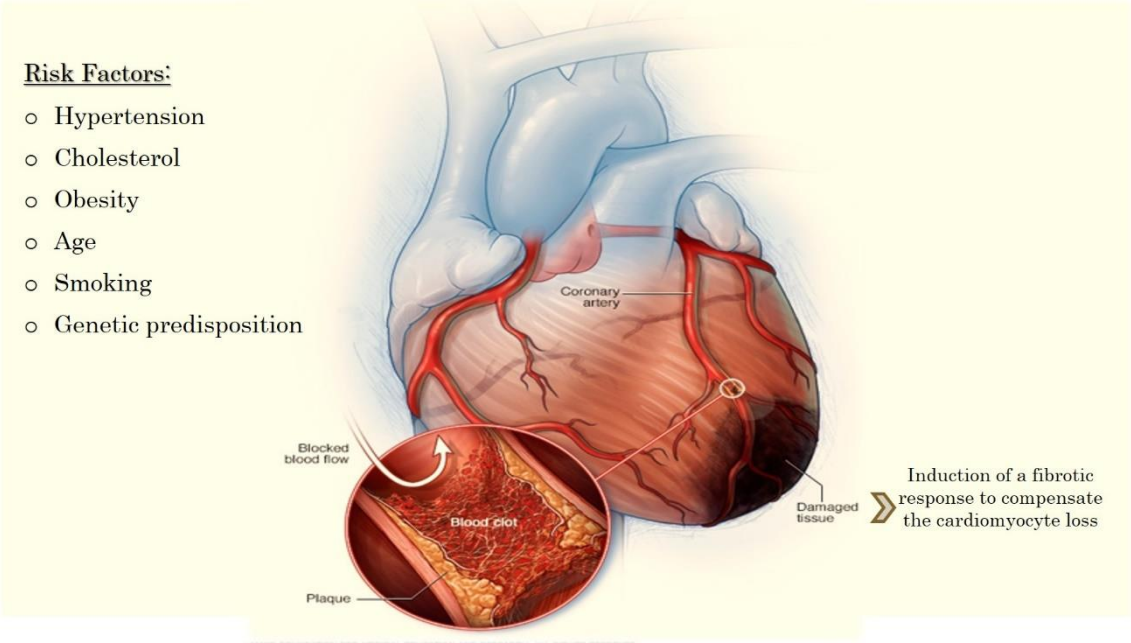

Figure 1.1 – Representation of the coronary artery disease (CAD) ultimately progressing into myocardial infarction (MI). The CAD consists in the accumulation of atheromas in the coronary blood vessels and several factors contribute to the formation of the atheromas plaques (hypertension, obesity, cholesterol and others). This disease, when in advanced stages can originate a MI due to the total occlusion of the vessel by the atheroma. A MI is characterized by tissue necrosis since the downstream tissue to the occlusion becomes oxygen and nutrient deprived. This damage is counteracted by the deposition of fibrotic tissue to maintain the heart integrity. However, this scar tissue does not have the same functions and structure of the native myocardium, and over-time can deteorate the heart condition, leading to heart failure. Image adapted from Mayo Clinic to medical educational purposes.

1.2. Current therapies versus new therapeutic strategies

Currently, there are several surgical procedures to treat CAD before it progresses to MI. The most common interventions are coronary balloon angioplasty (with the placement of a coronary stent) and coronary bypass. The first procedure simultaneously removes the plaques that occlude the arteries and places a biocompatible stent that allows the vessels to remain wide, whereas the second uses a vein from the body to substitute the portion of the occluded coronary artery[4,10]. Nevertheless, these procedures have a limited effect on long-term survival and do not prevent recurrence of symptoms [10]. Furthermore, for patients that already suffered from MI and developed heart failure, the only possible treatment is heart transplant, which is not always a sustainable solution because of organ supply paucity and immune rejection risks/problems[19–22], as well as patient ineligibility for heart transplantation due to comorbidities or advanced disease progression[21,23].

As a result of the high number of patients either in the heart transplant waiting-list or the ones that are not qualify for such procedure, new treatments are being researched and developed in order to regenerate the infarcted area. Such can be achieved by trying to

retrieve the lost cardiomyocytes as well as coronary vessels[24], since promoting neo-angiogenesis in the injured cardiac area is essential to allow the supply of nutrients and oxygen to cells, thus increasing the probability of cell/tissue regeneration[24,25]. So far, the most promising strategy to regenerate the lost cardiac cells relied in the used of stem cells (SCs) [25], since it was a longstanding dogma in the cardiac field that mammalian heart, including the human heart, was a post-mitotic/terminally differentiated organ without the ability to self-renew[26–29]. On the other hand, stem cells are undifferentiated cells capable of both clonogenic self-renew and differentiation into multilineages under appropriate conditions and specific signals, potentially originating different and functional cell types and consequently providing a cell source to repair several heart diseases, including MI[24,30]. Multiple studies/clinical trials have been performed employing either pluripotent stem cells or adult stem cells (originated from different tissues) to assess their potential in heart regeneration, and some trials exhibited promising initial results, with a reduction in the cardiac remodelling and improvements in the left ventricular ejection fraction (LVEF)[24,25,31,32]. Nevertheless, in long-term analysis some complications emerged such as the poor engraftment of the transplanted cells and the production of malignant cardiac rhythms resulting from the engraftment of electrically heterogeneous cells, not fully restoring the normal heart conditions[33]. Furthermore, based on these clinical trials results researchers began to speculate that the observed clinical benefits/improvements were not caused by *in vivo* differentiation of stem cells into cardiac cells, as initially thought and expected, but rather by the secretion of paracrine factors from these cells (such as cytokines, chemokines and/or growth factors). In heart therapy, paracrine factors seem to have a myriad of functions, from promoting angiogenesis, inhibiting cardiac cell apoptosis and fibrotic processes, enhancing cardiac muscle contractibility, as well as activating endogenous circulating or site-specific stem cells with repair mechanisms, which could explain the clinical improvements observed in the clinical trials[20,25,34].

As the research concerning the use of stem cells for cardiac regeneration persisted, it was recently discovered that the adult heart, unlike previously thought, possesses an endogenous cell pool, in a quiescent state, with stem-like properties that become active, upon injury to replace the cell loss[27,28]. These cells are denominated Cardiac Progenitor Cells (CPCs) and constitute a heterogeneous population of cells that populate different heart areas (niches)[20,35], capable of differentiating into cardiomyocytes, smooth muscle cells and endothelial cells[29,36,37]. Furthermore, this heterogeneous population of

progenitor cells has been further classified in subpopulations depending on their expression markers, for example there are the $Sca1^+$ CP cells, c-kit^+ CPCs and cardiosphere cells (CDCs). A few phase-I clinical trials using different subpopulations of CPCs have already been performed presenting favourable results, but it is still too early to extract any conclusion[21,25,38]. Still, a few questions regarding these progenitor cells need further enlighten, such as the limited cardiac regeneration after injury and if it is possible to enhance the mobilization of the remaining native CPCs to stimulate in vivo regeneration[34]. Several hypothesis partially explain why these cells are not able to counterattack the cardiac damage in vivo, from an insufficient number of CPCs remaining in the heart, which could also be aggravated by age-related CPCs losses, to a limited translocation ability of CPCs, the formation of immature cardiomyocytes, and even the extent of the insult and the pro-fibrotic environment, that all together could deeply impact on CPCs potentials[29].

Nevertheless, associated with CPCs existence or not, new published data suggests that mammalian cardiogenesis process can occur in the short period after birth, upon injury. This regenerating window is characterized by increased cardiomyocyte proliferation and robust angiogenic growth, despite some scarring still be present. In fact, in the work developed by Porrello *et al.*, it was described that neonatal mice hearts were able to regenerate the myocardium until seven days postnatal, but the regenerating ability sharply declines after such period[39,40].

Thus, further studies must be conducted as stem cell-based cell therapy is still not a fully understood process, requiring various optimizations and controls to allow the proper analysis of the results. Nevertheless, so far, stem cell therapy has proved to be safe and a promising treatment source.

1.3 From the Heart to Cardiac Fibroblasts (CF)

1.3.1 From the embryo to heart formation

The formation and development of the human embryo begin with the fusion of the male and female gametes, a process commonly designated by fertilization. From this process results a fertilized cell – the zygote – which within 24h after fertilization will undergo a series of mitotic cell divisions called cleavage. These cell divisions subdivide the large

embryo into smaller daughter cells designated blastomeres, thus allowing the embryo to grown in cell number but maintaining its size. By the fourth day after fertilization, the embryo reaches the 16-cell stage and is now designated by morula (Figure 1.2). At this point, cells have already started to reorganize, by the compaction process, to enable the segregation of cells into the peripheral outer cell layer group (trophoblast) that will be the source of the placenta components and associated extraembryonic membranes; and the central inner cell mass (ICM) group, also named embryoblast as it will give rise to the proper embryo(Figure 1.2). When the morula/embryo is at the 30-cell stage, it reaches the uterine cavity and develops a fluid-filled cavity which causes the embryoblast to form a compact mass at one side of the cavity (the embryonic pole), whereas the trophoblast organizes into a thin single-layered epithelium[41]. At this point, the embryo transforms into a blastocyst and at the 100-cell stage, around the seventh day after fertilization, it starts to adhere to the uterine wall been fully implanted in the endometrium around the ninth day. In parallel to the attachment process into the uterine wall, the embryoblast splits into two layers, the epiblast and the hypoblast (or primitive endoderm), that together constitute the bilaminar embryonic disc (Figure 1.2). On the fifteenth day of development the primitive streak (PS) is formed within this disc. The PS consists of a midline groove thickening along the midsagittal plane of the embryonic disc and, besides defining the three body axes, it also marks the beginning of the gastrulation process.

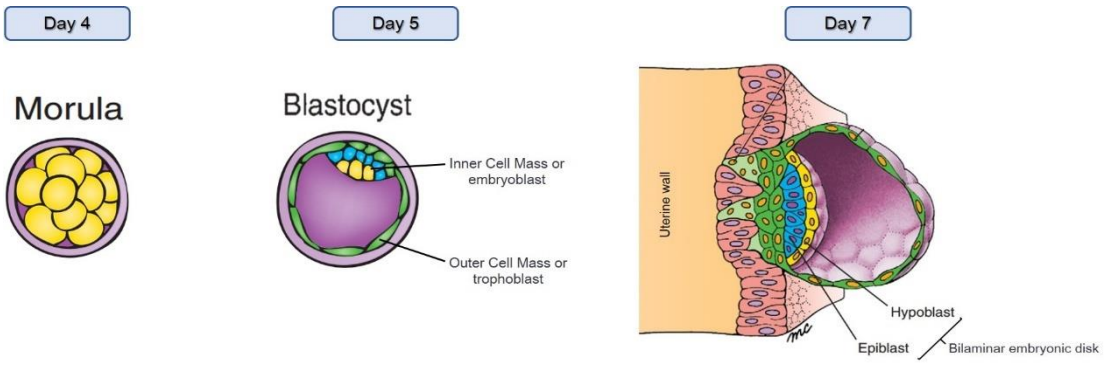

Figure 1.2 – Developmental stages of the zygote during early embryogenesis. After fertilization, the zygote suffers successive divisions becoming a morula at the fourth day of development and a blastocyst at the fifth day of development, after reaching the uterine cavity. Between the morular and blastocyst stages, the cells start to reorganize in the Inner Cell Mass (ICM) group, that latter gives rise to the proper embryo, and the Outer Cell Mass group, which is the source of placenta and extraembryonic membranes. Around the seventh day of development, the blastocyst contacts with the endometrium and begins to implant. In parallel to the attachment, the ICM group splits into the epiblast and hypoblast (or primitive endoderm) layers, together constituting the bilaminar embryonic disk. Image adapted from Larsen`s Human Embryology, 4thedition.

The gastrulation is a fundamental event for the formation of the three germinative layers (endoderm, mesoderm and ectoderm), in which epiblast cells take a huge part (Figure 1.3). During gastrulation, there is a migration of epiblast cells, resulting in some epiblast cells invading the hypoblast, displacing and replacing the existent cells to form the endoderm layer, whereas other epiblast cells migrate into the space between the nascent endoderm and epiblast to constitute the intraembryonic mesoderm[41] (Figure 1.3). Once the endoderm and mesoderm layers are established the epiblast cells stop their migration through the PS and give rise to the ectoderm.

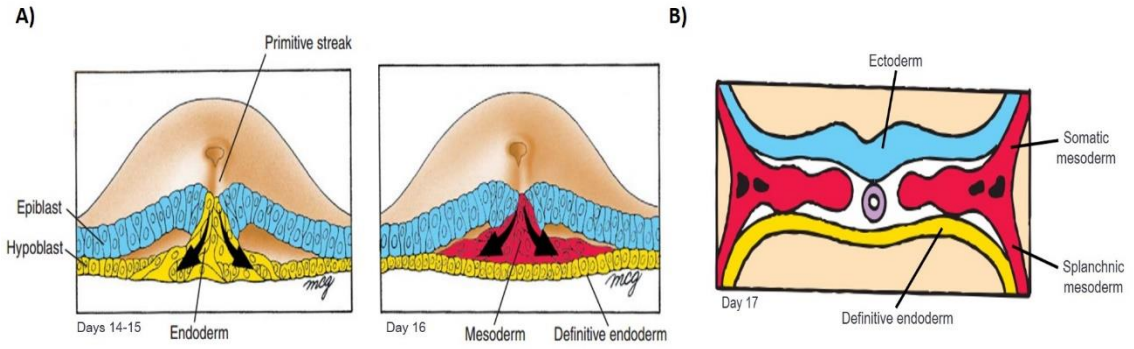

Figure 1.3 – Gastrulation process and formation of the lateral splanchnic mesoderm. A) The gastrulation process is essential for the formation of the three germinative layers (endoderm, mesoderm and ectoderm) and begins with the migration of the epiblast cells along the primitive streak (PS). The first wave of migrating epiblast cells displaces and replace the hypoblast cells to form the definitive endoderm, at the fourteenth day of development. Then, the subsequent ingressing cells migrate into the space between the epiblast and nascent endoderm layers, to constitute the intraembryonic mesoderm at the sixteenth day of development. Once the endoderm and mesoderm layers are fully established the epiblast cells stop their migration through the PS and originate the ectoderm layer. B) At the seventeenth day of development the lateral plate mesoderm splits into the dorsal somatic mesoderm and the ventral splanchnic mesoderm, which originates the heart. Images adapted from Larsen`s Human Embryology, 4thedition.

- <u>From lateral plate mesoderm to mature Heart</u>

Once the intraembryonic mesoderm layer is established, the cells reorganize and subdivide into four main groups, cardiogenic mesoderm, paraxial mesoderm, intermediate mesoderm and lateral plate mesoderm. At day 17 of development, the lateral plate mesoderm further splits into the splanchnic mesoderm, a ventral layer associated with endoderm, and into the somatic mesoderm, a dorsal layer associated with the ectoderm (Figure 1.3). It is from the splanchnic lateral plate mesoderm that the heart is originated been the first formed and functioning organ in humans. Initially, the embryonic heart consists of a single tube composed of contractile myocardium surrounding an inner endocardial tube with extracellular matrix, and despite still been in development, it starts

beating rhythmically at day 22 of development and at days24/25 is already pumping blood[41].

The embryonic heart starts to form due to cues from the adjacent layer that promote the mesoderm-derived cardiac progenitor cells (CPCs) to leave the PS, where they were originated, and migrate to cranial-laterally to either side of the streak. These cardiac precursors cells then form a cardiac crescent (CC, or primary heart field) within the splanchnic mesoderm[41] (Figure 1.4). As the embryo lateral folding process begins, the right and left sides of the CC fuse in the midline leading to the formation of a pair of vascular elements – the endocardial tubes – within each limb of CC. The process of formation of endocardial tubes is denominated by vasculogenesis and is triggered by signals of the underlying endoderm that directs a subset of cardiac crescent cells to an endothelial/endocardial lineage, consequently forming the tubes. As the folding process continues, both limbs of CC join and the lateral endocardial tubes coalesce into a single tube forming a single primitive heart tube, in the thoracic region, that harbours progenitors for the atria and ventricles and endocardium[41] (Figure 1.4). By day 21 of development, the primitive heart tube consists of an endocardial tube surrounded by an acellular layer of cardiac jelly produced by the myocardial cell layer, which derived from a mass of splanchnic mesoderm and encloses the endocardial tube. Simultaneously to the proliferation of myocardial cells within the primitive heart tube, cardiac progenitor cells from the second heart field (SHF) located both at the cranial and caudal pole (outside the original CC) are being recruited to lengthen the primary heart tube and, ultimately, will contribute to venous and arterial pole as well as to the right ventricle[41] (Figure 1.4). Then, at day 23 of development, while the primitive heart tube is still lengthening, the cardiac looping occurs, a crucial step that brings the four prospective chambers of the heart into the correct spatial localization in relation to each other. The last stages of heart development consists mostly in the remodelling of these chamber, developing of the septa and valves between them; formation of the epicardium, coronary vasculature and cardiac innervation and conducting system, along with the generalized maturation of cardiomyocytes and growth and differentiation of the other cardiac cells, culminating in the development of the functional four-chambered heart[42].

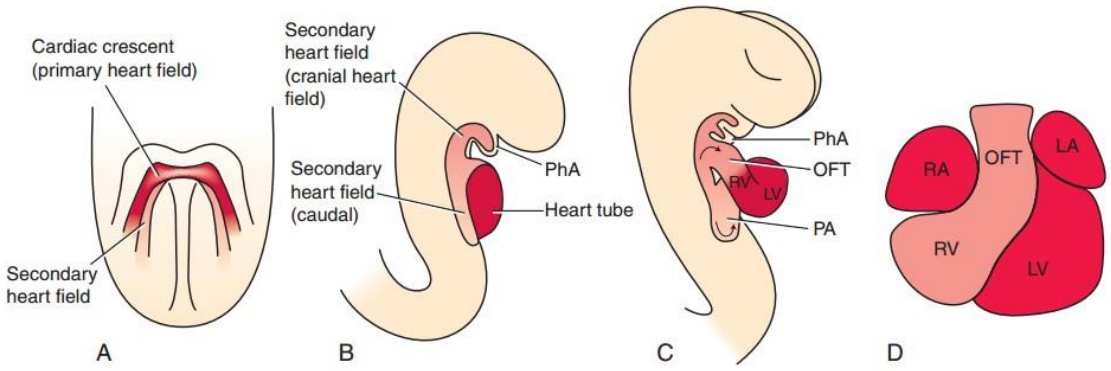

Figure 1.4 – First and second heart fields and their contribution to the mature heart. A) Locations of the cardiac crescent (CC) and secondary heart field (SHF, shown in light red in A-D). B) After formation of the primitive heart tube (derived from the folding of the CC and coalescence of the endocardial tubes into a single tube), cardiac progenitor cells from the SHF located at the cranial and caudal pole of CC are recruited to lengthen the primitive heart tube. C) The cardiac looping process occurs at the twenty-third day of development, after the heart tube had already started beating, and while it is still lengthening. D) Ventral view of the looped heart showing the contributions of the primary and secondary heart fields. The abbreviations used in the figure are left atrium (LA), left ventricle (LV), outflow tract (OFT), pharyngeal arch (PhA), primative atrium (PA), right atrium (RA), and right ventricle (RV). Image adapted from Larsen`s Human Embryology, 4thedition.

1.3.2 Heart cells

The adult/mature mammalian heart is composed of an acellular component, the extracellular matrix (ECM), and a cellular component mainly constituted by myocyte cells (cardiomyocytes), vascular cells (stromal and endothelial cells) and cardiac fibroblast[13,43,44]. Cardiomyocytes are the cardiac muscle cells present in the myocardium and responsible for generating force leading to heart contraction and consequently pumping the blood throughout the body. Due to their role in the healthy homeostatic heart, CMs have always been considered the most important cardiac cells. Many studies have been performed to understand the contribution of these cells to the cardiac performance, and contrary to what was thought, despite comprising most of the heart volume, these cells only account for 25-35% of all cells[45,46]. This fact highlights the importance of the non-myocyte cell population to maintenance of cardiac health, as well as their role in cardiac disease. The most relevant non-myocyte cells are the vascular cells (especially ECs) responsible for lining the interior surface of blood vessels, and the cardiac fibroblast cells[47], interstitials cells in charge of producing ECM – the scaffold of the heart[43–45]. Although non-myocytes cells account for the bulk of cardiac cells, the cells ratios of these cells are not a consensus in the ECs and CFs, due to the lack of consistent and reliable cell markers. However, a recent study by Pinto *et al.* clarified this question by demonstrating that in the human heart, ECs are the most abundant cell type (64% of non-

myocyte cells), while CFs only represent a minor population (15% of non-myocyte cells)[46].

Accordingly, the following section is a critical description of the dual nature of this type of cells whose relevance for normal heart homeostasis was overlooked for many years.

1.3.3 Cardiac fibroblast

Fibroblasts are described as connective tissue cells with mesenchymal origin, lacking a basement membrane, which in case of cardiac fibroblasts is a distinctive feature regarding the other cardiac cells. Also, morphologically, fibroblasts are flat cells with fusiform shape and salient protrusions and present an extensive rough endoplasmic reticulum and prominent Golgi apparatus, which allows these cells to produce and secrete a variety of ECM components, such as multiple collagens types and fibronectin[13,43,44]. Nonetheless, fibroblasts from different tissues present specific features, especially in their gene expression profiles granting these cells a phenotypic plasticity. It is due to their dynamic nature that defining the cardiac fibroblast population with a specific cell-marker as proven to be challenging, because depending on their location (atria, ventricles and so on) and stage of heart homeostasis (injury or repair) different genes signatures are expressed by the different CFs subpopulations[45]. Currently, the most used markers to described and isolate cardiac fibroblasts are the fibroblast-specific protein 1 (Fsp1), CD90, vimentin and discoidin domain collagen receptor 2 (DDR2), being these last two markers the most employed[17,43,44]. However, these markers present the limitations of either not being specific to this cell type or do not characterize all CFs subpopulations. For instance, vimentin is a very sensitive marker, identifying all CF cells but also other cardiac cells, like endothelial and smooth muscle cells; DDR2, on the other hand, is a better specific CF marker but is only be expressed in a small percentage of these cells[48]. Since all of these markers are not only specific for all CF populations, the best strategy to identify CF is combining multiple markers[17]. Recently a group of consistent markers has been emerging for defining this cell population and its function in the heart, that besides including the typically used CFs markers, also includes novel markers such as TCF21, an epithelial-to-mesenchymal transition (EMT) marker that defines the transition of epicardial cells to cardiac fibroblast, and platelet-derived growth factor receptor tyrosine kinase-alfa (PDGFR-α.) also an epicardium EMT marker specifically expressed in cardiac fibroblast lineage[18,45,49].

Regarding the embryonic origin of cardiac fibroblasts, three sources are well documented (Figure 1.5). According to lineage tracing mapping experiments, the major source of cardiac fibroblasts during embryogenic development is the proepicardial tissue[18,50] contributing with 80% of all cardiac fibroblasts. These proepicardial cells migrate to the surface of the heart forming the epicardium[43–45,50–52] and undergo epithelial-to-mesenchymal transition to form the epicardial-derived cells (EPDCs) that invade the myocardial wall and finally differentiate into fibroblasts, contributing to the compact myocardium[50]. The other minor contributors to CFs pool during embryogenesis are the endocardial tissue that originates a subpopulation of CFs mainly located in the interventricular septum, right ventricle and cardiac valves (also through EMT)[45,52,53], and the neural crest cells which produce CFs specifically located in the great vessels within the outflow tract region of the heart and in right atrium[52,54].

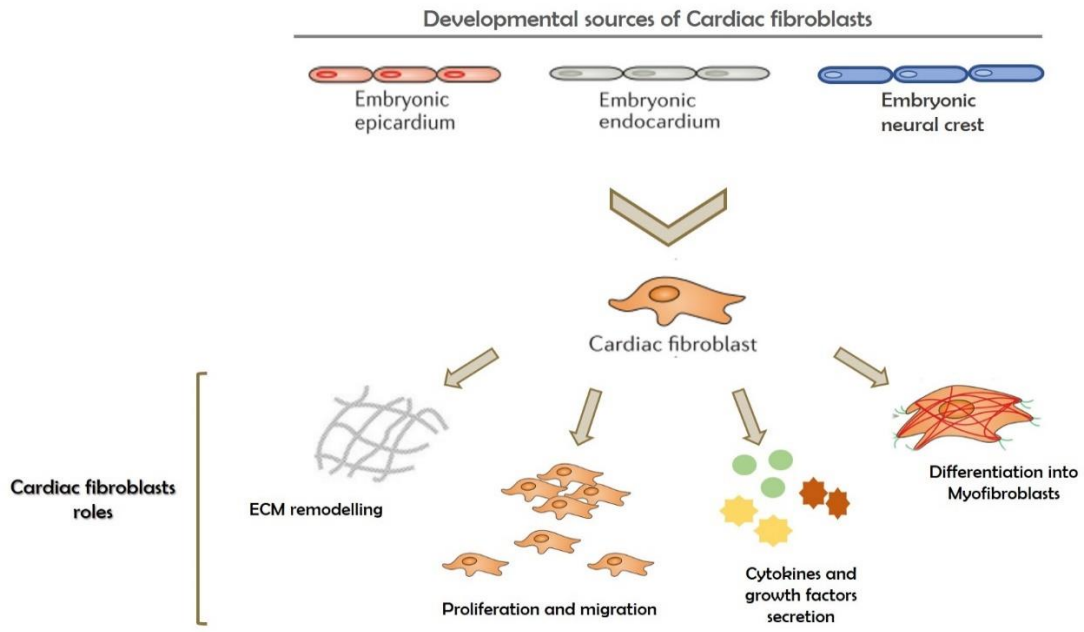

Figure 1.5 – Cardiac fibroblast developmental sources and roles in the adult heart (both in healthy and injury conditions). Tissue resident cardiac fibroblasts (CFs) arise from three documented embryonic sources, the epicardium (the major contributor), the endocardium and the neural crest. In the healthy heart, CFs have an important role in maintaining the extracellular matrix (ECM) remodelling, since it provides mechanical support to the other cardiac cells and distributes the mechanical forces throughout the myocardium. Other roles of CFs in the maintenance of the heart homeostasis are the secretion of paracrine factors that can induce the proliferation of cardiomyocytes or the formation of new blood vessels, for example. In the injured heart, CFs continue to be essential for the paracrine signalling, but also proliferate and migrate to injured area, and can also differentiate into myofibroblasts, which secrete high levels of ECM proteins to maintain the heart integrity after damage. Image based on representations of Tallquist *et al.*2017 and Souders *et al.*2009.

1.3.3.1 Cardiac Fibroblasts roles in the homeostasis of the heart

As described previously, CF cells are primarily recognised by their ability in forming and degrade extracellular matrix especially in the context of cardiomyopathies, originating scar tissue after a cardiac injury (Figure 1.5). Nevertheless, this ability as well as other biochemical and electrical properties of CFs are also essential to maintain cardiac homeostasis (Figure 1.5) [17]. In the healthy heart, the extracellular matrix produced by CFs provides mechanical support (scaffold) for all cardiac cell populations, besides contributing to the formation of specific heart structures, like the valves and the atrioventricular node. The ECM is an organized 3D stretch-sensitive network mostly composed by collagen fibres (mainly type I and type III), glycoproteins, proteoglycans. Besides, its structural function ECM also helps to distribute the mechanical forces throughout the myocardium, while conveying the mechanical signals to cells via surface ECM receptors, even co-coordinating the contractile movement of cardiomyocytes[17,43–45,55]. Since myocardial collagen is not a static protein and the cardiac tissue is continuously submitted to length and tension changes, it is important to sustain a balance between synthesis and degradation of ECM. This homeostasis is typically regulated by CFs upon the action of stimulators and inhibitors molecules but also the mechanical load, a critical modulator of CFs gene expression. Cardiac fibroblasts can sense mechanical stress signals via multiple transduction signal pathways that promote cell proliferation and upregulate the deposition of ECM proteins and cytokines and growth factors expression. Moreover, CFs also produce and secrete growth factors and other signalling molecules as a result of the interaction with other cardiac cells, like endothelial cells and cardiomyocytes [43]. As a matter of fact, it has been described that CFs promote CMs proliferation in normal conditions through the secretion of paracrine factors. In addition, CFs also interact directly with CMs through gap junctions (specifically connexin Cx43) and ions channels, contributing actively to the cardiac electrophysiological properties[17,43–45,55]. Since CFs are non-excitable cells and have a high membrane resistance, they are efficient mechano-electrical transducers, allowing the communication between myocytes that would be electrically isolated by connective tissue. This is an interesting feature as usually CFs, by themselves, would passively act as obstacles to the proper spread of electrical excitation[17,43–45,55].

Another important role of CFs, yet much unexplored, is the promotion of angiogenesis. The involvement of CFs in the angiogenesis process is known for several years, but there

are few studies on how fibroblasts contribute to the formation of blood vessels[43,56]. Although the exact process is unknown, it is thought that cardiac fibroblasts contribute to the angiogenic process by secreting angiogenic factors that assemble and stabilize endothelial cells, turning them into blood vessels[57]. These factors are fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), powerful angiogenic inducers along with matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) that have both pro- and antiangiogenic functions, according to environmental molecular cues[43]. New data from research involving the development of cardiac tissue patches has proved that support cells, such as CFs, are essential for the formation of EC sprouts in pre-vascularized tissue constructs. This research noted that CFs specifically provide good support for ECs to proliferate, leading to a high number of sprout formation, although still lacking to induce the formation of multi-cellular EC sprouts[58]. Some authors also suggest that for an optimal blood vessel formation, a direct interaction between cardiac fibroblasts and endothelial cells is necessary[57]. Since CFs appear to be a fundamental tool for the regeneration of injured cardiac tissue, not only by stimulating CMs proliferation, but also through the promotion of angiogenesis many researchers have beginning to investigate how CFs specifically can contribute to the cardiac angiogenisis.

In our group, we are interested in understanding if and how collagen and calcium-binding EGF domain-1 (CCBE1) expression in cardiac fibroblasts is involved in the promotion of cardiac angiogenesis, since this molecule is involved in the VEGF-C pathway. This topic will be further detailed below.

1.3.3.2 *CCBE1* and Cardiac Fibroblasts contribution to angiogenesis

The formation of the coronary vasculature is dependent on the expression of diverse angiogenic factors, being the most important factors belonging to the VEGF family. Particularly, VEGF-C appears to be fundamental for the development of the ventricular coronary vasculature, as disruption of this signalling molecule reduces the formation of coronary vasculature sprouts derived from the sinus-venosus[59]. Nevertheless, according to lymphatic development studies, the biosynthesis pathway of VEGF-C is dependent of another important molecule, the CCBE1 protein. Briefly, VEGF-C protein is produced as an inactive pro-peptide being proteolytically processed into its active form by CCBE1 complexed with A-disintegrin and metalloproteinase with trombospondin motifs3

(ADAMTS3)[60]. CCBE1, an ECM protein, has a dual role in the proteolytic processing of VEGF-C: it increases the processivity of ADAMTS3 and co-localizes this molecule with VEGF-C to favour the formation of the trimeric complex[61]. Therefore, CCBE1 is an essential protein to obtain a fully functional VEFG-C protein.

Most of the research on *CCBE1* gene is mainly focused in lymphatic development, since it is known that mutations in this gene cause the Hennekam syndrome, despicted by the lymphatic system malformation[62]. Notwithstanding, *CCBE1* is also being explored as an important regulator molecule in vascular formation since in lymphatic development this protein facilitates the budding and migration of lymphatic endothelial cells which give rise to the lymphatic vasculature. Furthermore, results from our group demonstrated that *CCBE1* is involved in the early cardiac commitment, influencing the migration and proliferation of cardiac progenitor cells [63]. Using a mouse embryonic KO *CCBE1* cell line it was possible to observe a decreased expression in the early mesoderm and *Islet1* cardiac progenitor genes, as well as, a low proliferation of these cells, assessed through the size of the embryoid bodies (EBs) [63,64]. Interestingly, patients with the Hennekam syndrome also present congenital heart defects (hypertrophic cardiomyopathy and ventricular septal defects), corroborating the contributing of CCBE1 to cardiac development. Besides been expressed during the early stages of cardiac development, our group has also proved the expression of *Ccbe1* in the proepicardium structure of the chicken heart[65]. In addition, recent results in *Ccbe1*-KO mice demonstrated that *Ccbe1* KO in embryonic hearts led to underdeveloped subepicardial coronary vessels, with high levels of accumulated immature VEGF-C[59]. This data highlights the importance of *CCBE1* for the VEGF-C production pathway and, consequently, in coronary vessels formation.

Since many results hint to the involvement of *CCBE1* in the cardiac angiogenic process, and CFs are characterized by the secretion of both paracrine angiogenic molecules and ECM proteins (which CCBE1 also happens to be), it is crucial to evaluate the role (and expression) of *CCBE1* in cardiac fibroblasts to understand if it is though CBBE1 secretion that CFs contribute to the angiogenesis, and if yes how the modulation of *CCBE1* in CFs affects the cardiac angiogenesis.

1.4 Human induced pluripotent stem cells (iPSCs) *in vitro* modelling

1.4.1 iPSC overview

In the regenerative field, stem cells are highly regarded as promising therapeutic agents, especially pluripotent stem cells because of their ability to originate cells of any of the three germ layers. Two examples of stem cells are Embryonic stem cells (ESCs) – obtained from the inner mass of the blastocyte – and induced pluripotent stem cells (iPSCs). iPSCs are mature somatic cells that have been genetically reversed to an embryonic-like state through ectopic expression of some transcription factors critical for the maintenance of the stemness properties[66]. The working hypothesis that the differentiated state of somatic cells is a reversible genetic change was firstly conceived in 1952 by Briggs and King, and years later by Gurdon. But only in 2006, Yamanaka and fellows got this breakthrough achievement. The investigators managed to induce the dedifferentiation of mouse fibroblast to a pluripotent sate using a mixture of four genes carried in a retroviral vector[67]. The successful experiment had a huge impact in the medical field, stimulating further research which resulted in many improvements and refinements to the Yamanaka method, from the types of cell used, to which techniques yield the best security and efficiencies of reprogramming. When successfully reprogrammed, iPSCs are self-renewal with the ability to differentiate into any human-specific cell type, similar to embryonic stem cells. Furthermore, iPSCs also resemble ESCs both morphologically, with round shape, large nucleolus and scarce cytoplasm, as well as molecularly expressing the same pluripotency markers[21,30].

These characteristics confer a great therapeutic potential to iPSCs in regenerative and personalized medicine offering an unprecedented potential to generate patient-derived iPSCs, creating a personalized offer to the demand (autologous treatment)[35]. Nonetheless, hiPSCs also present limitations that hampers their clinical application, the most concerning being the teratoma formation – germ cells tumours with cells from the three germ layers – which also happens using ESCs, since it's a characteristic feature of the cells' stemness. However, unlike ESCs, hiPSCs do no raise ethical concerns, since are obtained from adult tissue with minimal invasive methods, and neither provoke graft rejection upon transplantation as they are collected from the patient (autologous transplant)[9,28]. Other difficulties in using hiPSCs reside in the reprogramming process, that presents low efficiency, requiring the optimization of several variables before clinical

translation[68]. All the crucial steps in the hiPSCs reprogramming process will be explained hereafter.

Currently, hiPSCs are one of the most important investigation tools for *in vitro* modelling, allowing to replicate and study processes that occur *in vivo* during embryogenesis, elucidating cardiac diseases mechanisms' involving relevant gene mutations and how they affect the progenitors and cardiac cells[69]. In addition, hiPSCs also appear to be very promising in drug screening platforms for the development of new therapies [70]. In a recent publication, some researchers were able to create hiPSCs-derived vascular organoids and apply it to a drug screening pipeline[71,72].

1.4.2 iPSC – Reprogramming pluripotency from somatic cells

1.4.2.1 Cells sources

One of the most important aspects to consider in the generation of iPSCs is the selection of the cell source as starting material, since it will influence the whole reprogramming protocol. Differences in efficiency of iPSC derivation from distinct sources have been attributed to the original epigenetic state of the adult somatic cell type and its requirement to go through multiple steps of de-differentiation (e.g. mesenchymal to epithelial transition) to reach a pluripotent state. Any human somatic cell can be used to induce pluripotency, nonetheless not all produce with the same efficacy of reprogramming pluripotent cells [73]. Desired sources of somatic cells should be easily accessible, non-invasive, do not present the risk of immune rejection as a potential transplantation treatment and its reprogramming process ideally highly efficient[74]. Many somatic cell types have been successfully reprogrammed to iPSCs, being fibroblasts cells the most commonly used because of their accessibility[74]. However, due to its invasive procedure, different cell sources are gaining popularity, such as squamous cells from urine, blood cells and even keratinocytes[75]. In this section we will discuss the favouring points and the difficulties of using each of the different sources of cells (Figure 1.6):

Fibroblasts

Fibroblasts were the first cells to be reprogrammed in the breakthrough work performed by Yamanaka and colleagues[67]. Reasons for the common use of this cells include their cheap and easy handling *in vitro* culture conditions, their well description in the literature,

low methylation in the *NANOG* and *OCT4* genes, favours its reprogramming. Fibroblasts also exhibit a very high proliferation rate in the minimum medium. However, this can also be a disadvantage because the fibroblasts that were not reprogrammed can grow more than the reprogramed ones, making it difficult to purify the reprogrammed fibroblasts[75]. Other difficulties associated with the used of fibroblast are the long time needed to reprogram[76], about three to five weeks, the very low efficiencies of reprogramming, 0.01%-0.5%[75,77], and the process used to obtain the fibroblasts – skin biopsy. A procedure that is very painful to the donor, requiring an anaesthetic and with possible secondary effects[75,77].

Urinary cells

Squamous cells are exfoliated renal epithelial cell that every day detach from the tubular system and urinary system's organs[78], making it readily available and accessible to be collected from urine samples, without the need of medical interventions (non-invasive)[79]. This cells although being excreted, they are easily isolated[73] and still fully functional for reprogramming purposes. Normally, the process of iPSCs generation starting from urinary cells only takes two weeks[79] and yields high reprogramming efficiencies, lying between 0.1% and 4%[74,75].They can also be freeze and thawed, while continuing to be stable. Nevertheless, the reprogramming efficiency seems to decays after fiver passages[73].

Blood Cells

iPSCs derived from blood cells can be obtained from two sources, umbilical cord blood or adult peripheral blood[76].

In the case of **adult peripheral blood** two types of cells can be used to generate iPSCs, mobilized $CD34^+$ blood cells or mononuclear cells. In the first case, prior to the collection of the cells, it is necessary to pharmacologically stimulate the production and mobilization of the $CD34^+$ hematopoietic stem cells in the donor[76]. Usually, it is used the granulocyte colony-stimulating factor (G-CSF) to increase the quantity of hematopoietic stem cells in the peripheral blood. The collection of these cells is then performed while the blood of the donor flows through a machine. The main disadvantages of this technique are the time-consuming process, the cost associated with it and the health risks for the donor (pain, nausea, fatigue) [75,80]. The isolation of specific mononuclear cells is simpler and only consists in a density gradient centrifugation and posterior

purification of the cells. Nevertheless, in both cases, the reprogramming efficiency is only 0.01%[77], which could be non-adherent and slow-cycling properties of these cells[81].

Umbilical cord blood (UCB), more specifically the endothelial cells that cover the blood and lymphatic vessels, present a clear positive aspect when compared to adult somatic cells. Unlike adult somatic cells that have been accumulating mutations over a lifetime as the result of aging, umbilical cord blood cells are newborn cells that have fewer mitochondrial and nuclear mutations in their genome[81,82]. Moreover, the collection procedure of UCBs is very simple, without any risk for the donor, and since the cells are immunologically immature, there is not the need for a perfect HLA (*human leukocyte-antigen)* compatibility between the donor and the patient[83]. Although the isolation of endothelial progenitors cells from blood cord seems to be ten times higher than from adult peripheral blood[84], and thus providing higher numbers of primary cells to be reprogrammed to a pluripotency state, the process of harvesting and conservation this cells is very expensive and can only be performed immediately after birth. Furthermore, the efficiency in the reprogramming of this type of cells is very low, <0.01%, making it difficult to be successful and most commonly used[81].

Keratinocytes

Keratinocytes are keratin dense cells, present in the epidermis, and in hair follicles. There are two approaches to obtain keratinocytes for reprograming back to a pluripotent state: performing a skin biopsy (to isolate the epidermal keratinocytes) or simply by plucking strands of hair, ensuring the presence of the hair root[75,85]. The last approach is more appealing since it is an accessible and non-invasive route to obtain keratinocytes, presenting similar results to those obtained with a skin biopsy[85]. The use of keratinocytes to generate iPSCs presents several advantages. Keratinocytes have the capacity to proliferate in serum-free and low-calcium medium[75,85,86], which exerts a selective pressure on cells that do not proliferate on it, allowing that only the keratinocytes grow and maintain an undifferentiated state, facilitating the purification protocol. Moreover, reprogramming of keratinocytes only takes one to two weeks and presents efficiencies around 1-2%[75,85]. Nevertheless, the approaches to obtain the keratinocytes cells are somewhat invasive procedures[85], and the reprogramming procedure should be performed at lower passages, when the cells present a higher proliferation rate[75].

1.4.2.2 Pluripotency induction - delivery techniques

The induction of pluripotency in somatic cells can be normally achieved by the ectopic expression of transcriptional factor genes responsible for controlling the pluripotent state of cells. The most used transcription factors are *OCT4* and *SOX2*, combined with either *KLF4* and *c-MYC*[67,87] or *NANOG* and *LIN28*[88]. *OCT4* and *SOX2* are two essential factors in the upregulation of genes associated with pluripotency, namely *Utf1* and *Fgf4*, while silencing genes responsible for the differentiation of the cells[87,89]. To increase the reprograming efficiencies, addition to or substitution of the transcriptional factors is performed, but the use of small molecules, microRNA and culturing conditions can also improve the reprogramming efficiency[70].

In addition to the different reprogramming factors and sources of cells used to generate the iPSCs, it is also important to choose the right delivery method for the reprogramming factor enter efficiently into the cells. Two main delivery methods can be distinguished: Integrative methods or non-integrative methods, which include non-integrating vectors, excisable vectors or DNA-free approaches (Figure 1.6) [77].

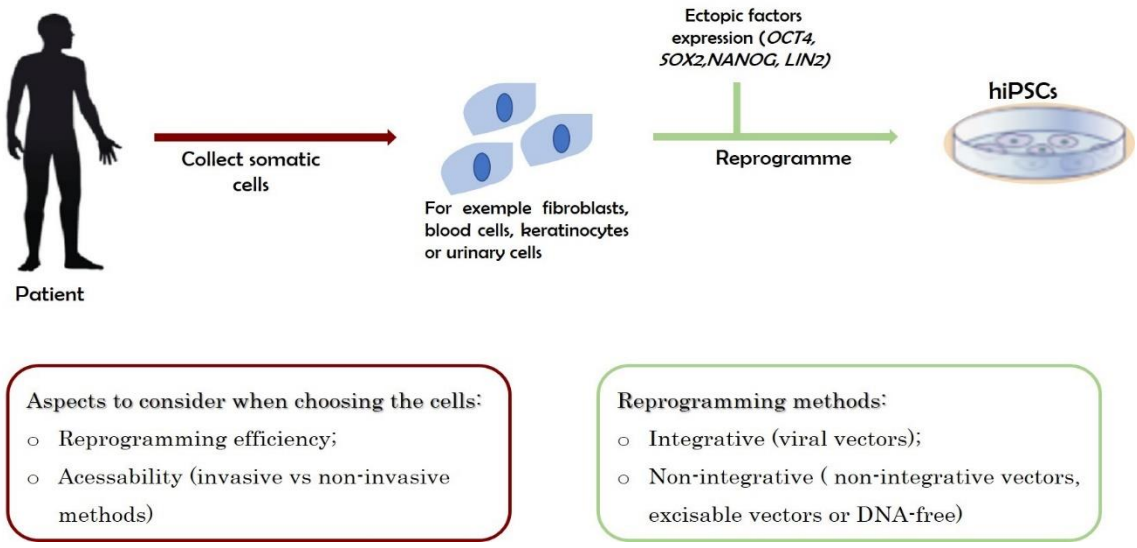

Figure 1.6 - Overview of the hiPSCs reprogramming process. First, cells are collected from the patient and then reprogrammed in vitro to a pluripotent state. After that cells can be differentiated into any cell type.

Integrative methods are mainly characterized by a stable and random integration of the vector along with the reprogramming factors into the host genome. The most used viral integrating vectors are retrovirus and lentivirus vectors. They both encode for RNA that is posteriorly reverse-transcribed to DNA, integrating the genome and passed to the

descendent cells. Retroviral vectors, which can be either replication competent or defective, have been extensively used in the early stages of clinical trials but currently, its use is limited to research purposes. In the iPSC research replication-defective retroviral vectors are the only ones used because they do not cause the lysis and death of the cells, they only deliver the material carried[90]. The major issue with the retroviral vectors is the fact they only transduce into cells in active division, having an efficiency of reprogramming between 0,01%-0,5%[77].On the other hand, the lentiviral vectors can infect both dividing and non-dividing cells[90], and are also better suited to the expression of polycistronic cassettes, allowing the insertion of all reprogramming factors at once, thus having an increased efficiency of reprogramming, between 0,1%-1%[77]. Once both vectors lead to persistent expression of the transgenes in the host cells they may cause insertional mutagenesis or chromosomal aberrations, transforming the host cells and be linked to tumorigenicity risks[77,90]. Taking this into account, this technique is not the appropriate choice in the regenerative medicine field, however, in the research field, it is quite useful, since, in general, the integrative vectors allow for a high reprogramming efficiency when compared to non-integrative methods[77].

Due to the drawbacks of the integrating vectors, safer methods that do not involve the permanent integration of the vectors into the host genome were developed in the last years. These methods include: non-integrating vectors, excisable vectors or DNA-free approaches[77,90].

The most used non-integrating vectors are episomal plasmids, Sendai Virus and adenovirus. Episomal plasmids are self-replicating vectors, maintained in the cells, as long as a selective drug pressure is being exerted. Unlike the conventional plasmids, episomes are stably expressed for a longer period (of time), but its reprogramming efficiency is also low[90]. Sendai virus is RNA-virus without a DNA intermediate phase, therefore not integrating into cells' genome. Nowadays, these viruses are widely used since they can infect all types of cells and produce large amounts of proteins. However, sometimes, Sendai virus can persist in the cells for at least 10 passages and the use of high temperatures are needed to eliminate its presence from the cells[90]. Contrary to Sendai Virus, adenoviruses are DNA viruses, however, like episomal plasmids, its genetic material is maintained in the cells as an extrachromosomal element. All these vectors can infect both dividing and non-dividing cells. However, this type of vectors has a much lower reprogramming efficiency (only 0,001%) than the integrating vectors, due to the

transient expression of the pluripotency factors, not allowing the complete epigenetic remodelling[77]. Especially in the case of adenoviruses that are cleared in the dividing cells, despite having a high transduction efficiency[90]. Nevertheless, it is being explored the conciliation of this type of vectors with small molecules to enhance the reprogramming efficiency[90].

Since completely non-integrative vectors have such low efficiency in the pluripotency reprogramming[69,91], researchers started to investigate the use of excisable vectors in this context. The excisable vectors have the advantage of, upon signalling, they can be excised from the host genome, being there only for a short period[77]. An example of excisable vectors are the transposons, and in this context, specifically the piggyback transposon. This is a host-independent, class II transposon that can be excise of the host genome by the brief expression of transposase enzyme. Despite being easily removed from the genome and having a very simple procedure, that can be applied to a variety of somatic cells, there are some concerns in the transposition process and how it can affect the cells since the insertion in the cells cannot be controlled[90]. This system was already applied to human embryonic fibroblasts demonstrating a moderate efficiency[69,77,91], but having a higher efficiency than non-integrating vectors.

Finally, the DNA-free strategy consists in the use of either proteins, small chemical molecules or the use of RNA molecules to reprogramming somatic cells to an ESC-like state pluripotency. The use of proteins or chemical compounds has demonstrated to have low reprogramming efficiency[77,92], requiring complex culture conditions and longer culture times[90] and, in the case of chemical molecules it may potentially alter the chromatin structure, inducing genetic or epigenetic abnormalities[77]. Proteins are also more difficult to obtain and purify in a reasonable quantity[93,94]. A more feasible technique is the use of RNA molecules to induce pluripotency. Despite being a more complex technically, it presents high reprogramming efficiencies, around 2%, without the risk of insertional mutagenesis or genomic integration[94]. To obtain the RNA encoding for the reprogramming transcriptional factors, firstly DNA plasmids encoding for those reprogramming factors are amplified by PCR (polymerase chain reaction). Once obtained the amplified fragments corresponding to the reprogramming factors, they are used as templates for RNA synthesis. Afterwards, the ssRNA is processed so it does not trigger an immune reaction, and to avoid being destroyed by the cell, since that is the fate of ssRNA. The modifications include the incorporation of 5'guanine cap into all RNA

molecules and phosphatase treatment. Finally, RNA fragments are transfected into the cells, usually associated to a cationic lipid delivery vehicles[94].

Summing up, for reprogramming strategies, gene-delivery methods are the most appealing techniques in comparison to protein transduction or chemical induction. Nowadays, despite the efforts to improve the non-integrating methods so it can be applied in medicine without having health risks to patients, the integrating methods are still the ones that offer a more efficient reprogramming of the pluripotent state in somatic cells.

Once the hiPSCs are reprogrammed, it is necessary to culture these cells in specific culturing conditions to select the colonies that were successfully reprogrammed, and further expand them so that the colonies cells can be characterized. The characteristics evaluated are mostly associated with the pluripotent phenotype of the cells, such as the expression of pluripotent markers, the morphology of the cells and their rate of proliferation.

1.4.3. CRISPRi in hiPSCs

In the biomedical and biotechnology fields, targeted genome engineering is a very common modification performed to cell lines, or even animals and plants. One of the technologies widely used is the clustered regularly interspaced short palindromic repeats (CRISPR) technology to induce targeted mutagenesis[95–97]. CRISPR technology was developed based on the defence system discovered in bacteria and archea, who use it as an immunity response against external nucleic acids, especially phages genomes[98]. Basically, the system consists in two small RNAs molecules fused together that will form a complex with the endonuclease Cas9, to specifically recognise (by complementarity) and target a sequence and consequently enable double-strand breaks in the DNA of the sequence's genomic region[98]. As the interest in this topic grew, the scientists began to understand the potential of this efficient and simple genome editing system[99]. Therefore, more research and adaptations to this system were performed, so nowadays the two independent small molecules of RNA were replaced by a chimeric form of guided-RNA which contains the important sequences of both RNAs fused in one RNA[99]. Moreover, it was also discovered different versions of the Cas9 protein, which confers versatility on this system, depending on the intended result. For example, it was developed a deactivated Cas9 (dCas9) protein to allow the activation or repression of a gene

expression, by fusing the Cas9 protein with regulatory domains, instead of conventional knockout of the gene which cannot be undone[97,99,100]. This late system with the dCas9 system is more generally referred as CRISPR interference (CRISPRi) system. The cell line used in this project is a hiPS cell line modified by CRISPR technology, to generate CRISPRi-dcas9 iPS cell line capable of precisely and efficiently induce the knock-down of *CCBE1* gene. The downregulation of *CCBE1* is a reversible process dependent of doxycycline, a molecule that activates the nuclease deactivated version of Cas9, associated with a Krüpple associated box (KRAB) repression domain, producing a highly efficient transcriptional interference[97,101]. The target specificity for *CCBE1* is provided by a single chimeric gRNA, coded in a vector. This system will allow us to study and understand the impact of the absence CCBE1 protein during different time points of CFs differentiation (Figure 1.7).

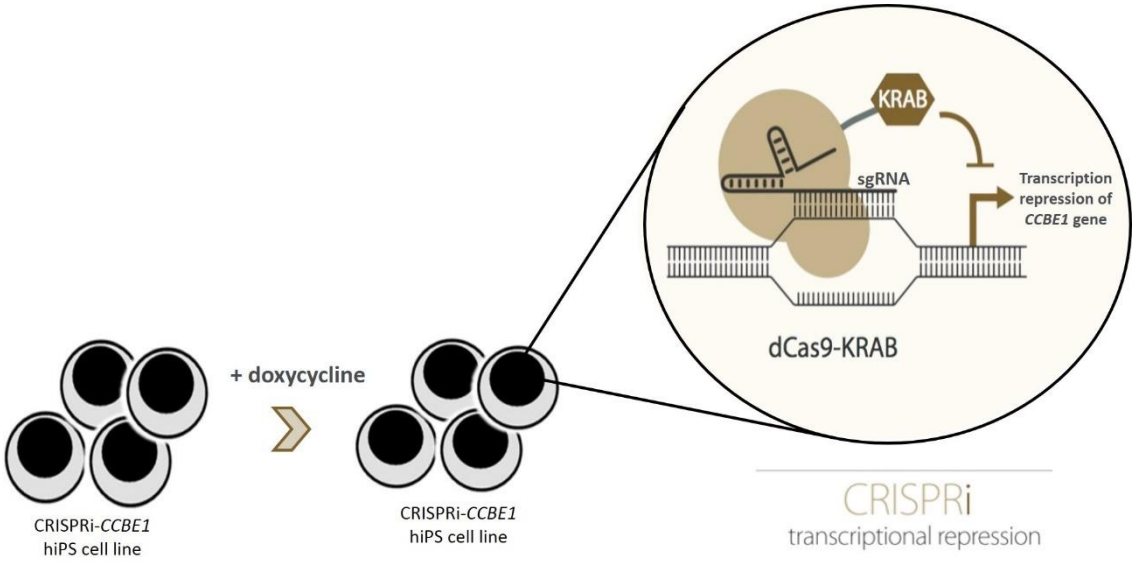

Figure 1.7 - Representation of the action mode of CRISPRi-CCBE1 knockdown, by doxycycline induction. In the normal cell medium conditions *CCBE1* gene is expressed normally, however after supplementing the cell medium with doxycycline, this molecule induce the expression of the dCas9-KRAB protein. This protein will in turn induce the expression of a *CCBE1* specific gRNA. The dCas9-KRAB protein associated with the gRNA will inhibit the expression of *CCBE1* by preventing the association of transcription factors to the gene. Image adapted from Horizon group.

1.5 Objectives

Many strategies have been developed to regenerate the heart after MI damage. One of the most promising approaches is the use of pre-vascularized patches, which not only supply cardiomyocytes to repopulate the damaged area, but also contain endothelial cells to allow blood vessels formation *in vivo*. However, besides endothelial cells, stromal cells, such as cardiac fibroblast, are also important and needed to promote the formation of new vessels through the secretion of angiogenic paracrine factors. Results from our laboratory indicate that the formation of the ventricular coronary vessels is influenced by the presence of VEGF-C and its interaction with CCBE1. In fact, inhibition of *CCBE1* expression was shown to reduce the formation of coronary vasculature sprouts derived from the sinus-venosus through an accumulation of immature pro-VEGF-C. Moreover, we verified that *CCBE1* is expressed in mouse embryonic cardiac fibroblasts. Therefore, we hypothesized that *CCBE1* expression and secretion by cardiac fibroblasts would trigger the development of new vascular networks from endocardial progenitors, suggesting a key role for cardiac fibroblast a vital role in regenerating blood vessels.

With this in mind, the major aims of this dissertation were:

- Establish a high yield differentiation protocol to generate CFs from hiPS cells;
- Evaluate the expression of *CCBE1* throughout the differentiation protocol;
- Modulate the *CCBE1* expression throughout the differentiation process to understand whether *CCBE1* inhibition affects the generation and morphology of the cardiac fibroblasts.

Chapter 2 – Materials & Methods

2.1 CRISPRi-*CCBE1* hiPS cell line

The cell CRISPRi-*CCBE1* KD hiPS cell line used in this thesis was already available in our lab, resulting from a PhD project of a former member (unpublished work).
Basically, a transgene containing a deactivated Cas9 nuclease (dCas9) fused with a Krüppel associated box (KRAB) repressor domain, was integrated into the adeno-associated virus integration site (AAVS1) locus of a wild type hiPSCs cell line, originating the CRISPRi hiPS cell line. This cell line contains doxycycline-inducible and high fidelity TetO promoter, as well as a mCherry reporter gene in the AAVS1 locus.
For *CCBE1* repression experiment, a vector containing a gRNA specific for *CCBE1* transcription start site (since the closer to the TSS more efficient the knock-down) and the mKate reporter gene were introduced into the CRISPRi hiPS cell line, generating the CRISPRi-*CCBE1* KD hiPS cell line.
Using this cell line, it was verified that we can obtain a reduction of the expression of *CCBE1* by 80%, in a pluripotency state, upon 7/8 days of doxycycline addition. Moreover, we verified that the addition of doxycycline to cells does not affect its stemness or proliferative state.

2.1.1 Cell culture

Handling of the cells, medium preparation and other techniques that require aseptic conditions were performed in laminar flow cabinet after irradiation for 15 minutes with ultraviolet (UV) light.

hiPSCs were maintained on 6-well-plates previously coated with GelTrexTM LDEV-Free (Gibco® Life technologies), a soluble matrix composed of laminin, collagen IV, entactin and heparin sulfate proteoglycans that promotes cellular adhesion and proliferation. To ensure the growth, expansion and pluripotency of hiPSCs, Essential 8^{TM} (E8) Flex medium (Gibco® Life technologies), a xeno- and feeder-free medium, was used, with daily changes of the medium. To do this, cells were passaged when they rich 70 - 85% confluence. At that time, hiPSCs were incubated at 37ºC and 5% CO2 for 5 minutes with DPBS1x (Gibco® Life technologies), followed by incubation with 700µl of Versene 1x (Gibco® Life technologies), also for 5 minutes at 37ºC and 5% CO2 to

detached cells. Next, the cells were resuspended mechanically with E8 medium supplemented with RevitaCell Supplement 100x (Gibco® Life technologies), and plated in a new GelTrex-coated well, usually at a 1:4 dilution, (corresponds to 30-40% of confluence). hiPSCs were maintained in a humidified incubator at 37º C, with 5% of CO_2 and routinely analysed under phase contrast microscopy (Evos XL Core (Thermo Scientific), to verify its morphology and confluence.

2.1.2 Differentiation of hiPSCs in Cardiac Fibroblasts (CFs)

In a monolayer culture, CRISPRi-*CCBE1* hiPSCs were differentiated into Cardiac Fibroblasts according to Bao *et al.*2017 protocol[102], which consists of a three phases process: first, the differentiation of hiPSCs to Cardiac Progenitor Cells (CPCs); second, the differentiation of CPCs to proepicardium cells; and finally, the differentiation of epicardium cells to Cardiac Fibroblasts. This protocol uses small molecules and cell passages to conduct the differentiation, having a total duration of 22days. Since the cell line used in our laboratory is different from the cell line of the original protocol, some optimizations to the differentiation protocol were made to increase the yield and efficacy of the differentiation in our cell line.

2.1.2 a) CPCs differentiation

hiPSCs were culture on GelTrex™-coated 12-well plate in E8 medium supplemented with 0.5µl/ml of RevitaCell. The initial seeding density for the differentiation protocol was $1.9x10^5$ cells per well. This day was designated day -3 of differentiation. At days -2 and -1 of differentiation the medium was replaced for fresh E8 medium however, on day -1 the E8 was preconditioned with 0.6 µM CHIR99021(Stemgent®).

On day 0, when cells reached 100% confluence RPMI B27/minus insulin Medium (also designated by RB^- medium) (Gibco® Life technologies) supplemented with 12 µM CHIR99021 was added to cells (2ml per well). In the next day, the medium was changed for fresh RB^-. This medium was maintained until day 3 of differentiation when a combined medium consisted of a mix of fresh RB^- with the medium in which cells were cultured and supplemented with 4 µM IWP4 (Stemgent®) was added to the cells and maintained until day 5 of differentiation. At day 5, medium was changed for fresh RB^-.

2.1.2 b) Proepicardium differentiation

On day 6 of differentiation cells were plated in new GelTrex^{TM}-coated 12-well plates. The cell passaging was performed by incubating cells for 4 minutes at 37ºC with PBS, followed by incubation with Tryple Select reagent at the same conditions, which completely dissociated cells from the matrix. Then, RB^{-} medium was added to neutralize Tryple Select (Gibco® Life technologies) effects. Next, cells were centrifuged at 1000 rpm for 5 minutes and the pellet resuspended in LaSR medium (composed of DMEM/F12 GlutaMAX^{TM} (Gibco® Life technologies) supplemented medium, 100µg/ml ascorbic acid and 2.5mM L-glutamine). Cells were counted in a Neubauer chamber and plated at a seeding density of 2.0x10^{5} cells per well of the new 12-well plate.
On days 7 and 8 of differentiation LaSR medium supplemented with 3µM CHIR99021 was added to cells (1ml medium per well). Daily medium changes with LaSR medium were performed until day 12 of differentiation.

2.1.2 c) CFs differentiation

On day 12 of differentiation cells were plated in GelTrex^{TM}-coated 24-wells plate. The cell passaging was performed by incubating cells for 9 minutes at 37ºC with PBS, followed by incubation with Tryple Select reagent for 10 minutes at 37ºC. Then, directly upon the dissociation reagent was added LaSR medium to neutralize its effects. Next, cells were centrifuged at 1000 rpm for 5 minutes and the pellet resuspended in LaFBS medium (DMEM/F12 + 1% Penicillin/streptomycin + 1%L-glutamine +10% FBS (Sigma)). Cells were counted in a Neubauer chamber and plated at a seeding density of 8-12 x 10^{4} cells/well.
From day 13 of differentiation until day 19 of differentiation, daily medium changes were performed with LaFBS medium supplemented with 10ng/ml of bFGF (Stemgent®).

2.1.2.1 *CCBE1* modulation through doxycycline

To perform the inhibition of *CCBE1* during the first six days of differentiation, doxycycline (Sigma) was added every day to the cells, at a final concentration of 2 µM.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from undifferentiated hiPSCs and differentiated hiPSCs at several time points of cardiac fibroblast differentiation protocol. For such TRizol Reagent (Invitrogen) plus Direct-zol™ RNA MiniPrep kit (Zymo Research) were used, according to the manufacturer's instruction. RNA quantification and quality ratios (260/280 nm and 260/230 nm) were determined with a spectrophotometer (NANODROP 2000, thermo Scientific) and the isolated total RNA stored at -80ºC.

1 µg of total RNA was used to synthesize cDNA with the RevertAid RT Reverse Transcription Kit (Thermo Scientific). Basically, the RNA was incubated with oligo(dT) primers and Nuclease-free Water (Ambion) at 65ºC, 5 minutes. Then, it was added a master mix solution containing: reverse transcriptase buffer, RiboLock RNase Inhibitor, 10mM dNTPs and 1U/µl of RevertAid Reverse Transcriptase enzyme. The samples were incubated at 42ºC, 60 min followed by 70ºC, 10 min to denature the enzyme. The cDNA samples were stored at -20ºC.

2.3 Real-time quantitative polymerase-chain reaction (RT-qPCR)

For qPCR, the cDNA samples were diluted 1:10 with Nuclease-free Water and 2 µl were used to quantify by qRT-PCR the expression of several genes. In addition to cDNA, the reactions mixture was composed by 7.5µl of SensiFast SYBR Lo-ROX (Bioline), 0.1µM of each forward and reverse primers listed in Table2.1. and 3.5µl of Nuclease-free water. The amplification and fluorescent quantification were obtained in ABI Quanti5 Real-time PCR machine, and the amplification program was as follow: pre-incubation at 95ºC for 2 minutes for initial denaturation of double-strand chains; 40 cycles of denaturation at 95ºC for 10s, annealing for 10s with the temperature specific for each primer pair, and extension at 72ºC for 20s;

The data were acquired during the extension phase. The level of target-gene expression was obtained through the $2^{-\Delta\Delta Ct}$ relative quantification method, normalized to GAPDH and β-actin housekeeping genes expression and using hiPSCs as reference/control sample. Each sample was performed in triplicate reaction and the results were normalized to the negative control sample, the hiPSCs sample.

Table 2.1 - Primers used for qPCR analysis, with the respective sequences and annealing temperatures

Gene	Primer Forward (5'→3')	Primer Reverse (5'→3')	Annealing Temperature (ºC)
NANOG	CATGAGTGTGGATCCAGCTTG	CCTGAATAAGCAGATCCATGG	55º
BRACHYURY	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG	56,9º
NKX2.5	CAAGTGTGCGTCTGCCTTT	CAGCTCTTTCTTTTCGGCTCTA	63º
ISLET1	AGATTATATCAGGTTCTACGGGATCA	ACACAGCGGAAACACTCGAT	56,9º
WT1	ATAGGCCAGGGCATGTGTATGTGT	AGTTGCCTGGCAGAACTACATCCT	56,7º
GATA5	ACCAAGATTCCCAGTGAAGCACCT	TCCGTCTATCCATGTGGGCAATGA	66º
TBX5	ACAAAGTGAAGGTGACGGGCCTTA	ATCTGTGATCGTCGGCAGGTACAA	63º
TBX18	TTAACCTTGTCCGTCTGCCTGAGT	GTAATGGGCTTTGGCCTTTGCACT	68º
TCF21	GAAGTGGTGACCGCGAGCCG	AGTGTTCTCGCGGGGTGGGA	66º
HAND2	CAAAATCAAGACCCTGCGCC	ATTTCGTTCAGCTCCTTCTTCC	56,7º
CCBE1	GCAAAAGATTGCTCTGCTCCC	GGAGGTCCTGGAAGGTAGGTG	61,3º
GAPDH	CTGGTAAAGTGGATATTGTTGCCAT	TGGAATCATATTGGAACATGTAAACC	61,3º
B-ACTIN	GCAAAGACCTGTACGCCAAC	AGTACTTGCGCTCAGGAGGA	55º

2.4 Immunocytochemistry (ICC)

2.4.1 Immunocytochemistry for pluripotency markers

For ICC, CRISPRi-*CCBE1* hiPSCs were cultured in a 24-wells plate and seeded at a density of $1.0x10^5$ cells/well in GelTrex™-coated coverslips. When cells reached 50-60% confluence, they were washed with PBS1x and fixed with 4% PFA (Sigma) for 30 minutes at room temperature (RT). Next, cells were washed again with PBS1x and permeabilized with 0.1% Triton X-100 (Sigma) in PBS1x at RT, for 30 minutes in case of Nanog and Oct4 markers, and only 7 minutes for SSEA4 marker since it is a cell surface protein and the permeabilization can disrupt its lipidic structure. Subsequently, to avoid unspecific antibody-binding, cells were incubated with Blocking Solution (BS: 0.4g BSA, 0.04g NaN3, 1.5g glycine in 200ml PBS1x) for 30 minutes at RT and incubated with the respective pluripotency primary antibodies (Table 2.2) overnight at 4ºC. The next day, cells were washed 3 times with BS, for 5 minutes each, and incubated with the secondary antibodies (Table 2.2) overnight at 4ºC, in the dark. Finally, cells were washed with BS

3 times, for 5 minutes each, and mounted in Vectashield (Vector Laboratories). The images were acquired in a widefield fluorescent microscope (Zeiss Z2 Axio Imager Microscope, Carl Zeiss).

2.4.2 Immunocytochemistry in differentiation

The ICC for proepicardium was performed at day 12 of differentiation for WT1 marker, whereas the ICC for cardiac fibroblast characterization was performed at day 19 of differentiation using VIMENTIN. To perform the ICC, on the cells were seeded at day 6 and day 12 of differentiation, respectively in GelTrex^{TM}-coated coverslips in a 24-wells plates.

Both ICCs followed the same protocol, first cells were washed with PBS1x and fixed with 4% PFA for 15 minutes at room temperature (RT). Next, cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS1x at RT, for 30 minutes, followed by an incubation overnight at 4ºC with Blocking Solution (BS: 0.4g BSA, 0.04g NaN3, 1.5g glycine in 200ml PBS1x to prevent unspecific antibody-binding. In the following day, cells were incubated with the respective primary antibodies (table 2.2) overnight at 4ºC. In the third and final day, cells were washed with PBS, and incubated with the secondary antibodies (Table 2.2) for 2h, at RT. Then, cells were washed once again 3 times with PBS for 10 minutes each, before mounting the coverslips in Vectshield (Vector Laboratories). The images were acquired in a widefield fluorescent microscope (Zeiss Z2 Axio Imager Microscope, Carl Zeiss).

2.5 Flow cytometry

Differentiated cells were collected and centrifuged at 2000rpm for 5 minutes. Cells were resuspended/washed in 1ml 1% BSA-PBS (Sigma) and centrifuged at the same conditions, followed by an incubation with PFA 4%, 15 minutes at room temperature (RT) to fix the cells. Without removing the PFA, cells were washed with 1ml 1% BSA-PBS and centrifuged at 2000rpm for 5 minutes. Next, cells were resuspended and incubated with saponin (diluted 1:10 with 1% BSA-PBS) (Gibco®LifeTechnologies) for 15 minutes at RT, to permeabilize the cell membrane and nucleus since the detection target was an intracellular marker. Again, cells were washed with 1ml 1% BSA-PBS and this volume divided in 3 different eppendorfs, which would be used for the unstained

control sample, the secondary antibody control sample and the primary antibody sample. All 3 eppenddorfs were then centrifuged and while the primary antibody sample was incubated with the primary antibody (NKX2.5 1:100 dilution), for 30 minutes, the other samples were resuspended in 1ml 1% BSA-PBS and kept on ice (4ºC). After the primary antibody incubation period, 1ml 1% BSA-PBS was added to the primary antibody sample and together with the secondary antibody control sample were centrifuged. Both samples were incubated with the secondary antibody (1:5500 dilution) for 30 minutes, at 4ºC, in the dark. Finally, all samples were centrifuged and resuspended in 225μl final volume of 1% BSA-PBS and transferred to the appropriate tubes. Samples were analysed on CantoII with 10000 events registered per sample. Primary and secondary antibodies used are listed in Table 2.2.

2.6 Statistical Analysis

Statistical Analysis was performed using GraphPad Prism 6 software for windows (GraphPad Software, Inc; San Diego California, USA). All the experimental data are represented as mean ± SD (standard deviation). The statistical test applied was One-way ANOVA Newman-Keuls multiple comparison test. The probability values considered statistically significant were $*p < 0.05$, $**p < 0.01$, $*** p < 0.001$.

Table 2.2- Primary and secondary antibodies used for either immunocytochemistry (ICC) or Flow Cytometry (CF) with the respective dilutions and manufactures

Antibody	**Supplier (catalog No.)**	**Dilution (application)**
Anti-NANOG rabbit polyclonal	Abcam (ab21624)	1:200 (ICC)
Anti-OCT4 rabbit polyclonal	Abcam (ab19857)	1:400 (ICC)
Anti-SSEA4 mouse monoclonal	Abcam (ab16287)	1:200 (ICC)
WT1 (C-19) rabbit polyclonal	Santa Cruz (sc-192)	1:50 (ICC)
Anti-Human Vimentin goat polyclonal	CHEMICON (AB1620)	1:25 (ICC)
Human Nkx2.5 goat polyclonal	R&D system (AF2444)	1:100 (FC)
Alexa Fluor® 594 donkey anti-rabbit IgG	JacksonImmunoResearchLabs (711-585-152)	1:300 (ICC)
Alexa Fluor® 488 donkey anti-mouse IgG	JacksonImmunoResearchLabs (715-545-151)	1:300 (ICC)
Alexa Fluor® 488 donkey anti-rabbit IgG	JacksonImmunoResearchLabs (711-545-152)	1:500 (ICC)
Alexa Fluor® 594 donkey anti-goat IgG	Life technologies (A11058)	1:500 (ICC)
Alexa Fluor® 647 donkey anti-goat IgG	Life technologies (A21447)	1:5500 (FC)

Chapter 3 – Results and Discussion

3.1 Evaluation of the pluripotent phenotype of the CRISPRi-*CCBE1* hiPS cell line

CCBE1 is an extracellular matrix protein that facilitates the conversion of immature VEGF-C into its active form, which in turn has an active role in angiogenesis. The current hypothesis is that *CCBE1* expression and secretion by cardiac fibroblasts trigger the development of new vascular networks from endocardial progenitors, hence giving cardiac fibroblast a vital role in the regeneration of lost blood vessels. Therefore, one of the goals of this work is to establish a high yield monolayer culture differentiation protocol to generate mature cardiac fibroblasts that can be posteriorly co-culture with endothelial cells to originate a 3D cardiac patch for coronary artery disease therapy purposes. Furthermore, we also propose to understand how *CCBE1* expression during cardiac fibroblast differentiation or in cardiac fibroblasts *per se* is involved in the coronary vasculature formation.

For that purpose, we took advantage of a hiPSCs CRISPRi-*CCBE1* knockdown cell line previously designed and generated in our laboratory. This cell line allows us to precisely and efficiently knockdown *CCBE1* gene at specific time points during the differentiation of hiPSCs towards a CF cell lineage and study the effect of its absence.

Before starting the differentiation experiments we re-confirm the pluripotent phenotype of the CRISPRi-*CCBE1* cell line. For the pluripotent assay experiment we analysed three commonly pluripotent markers, NANOG and OCT4 (both nuclear markers), and SSEA4, a surface protein marker. All three pluripotency markers are expressed in the germ layers of the developing embryo and while NANOG and OCT4 are transcription factors responsible for triggering a cascade of events resulting in the maintenance of cells pluripotency or self-renew, SSEA4 is stage-specific embryonic membrane bound glycolipid carbohydrate protein. As we can observe in Figure 3.1 CRISPRi-*CCBE1* hiPS cells presented nuclear staining for both NANOG and OCT4 while SSEA4 is present in the membrane of the cells as expected. With this assessment we ascertained the pluripotent phenotype of the hiPSCs, which will be used for *in vitro* differentiation towards CFs.

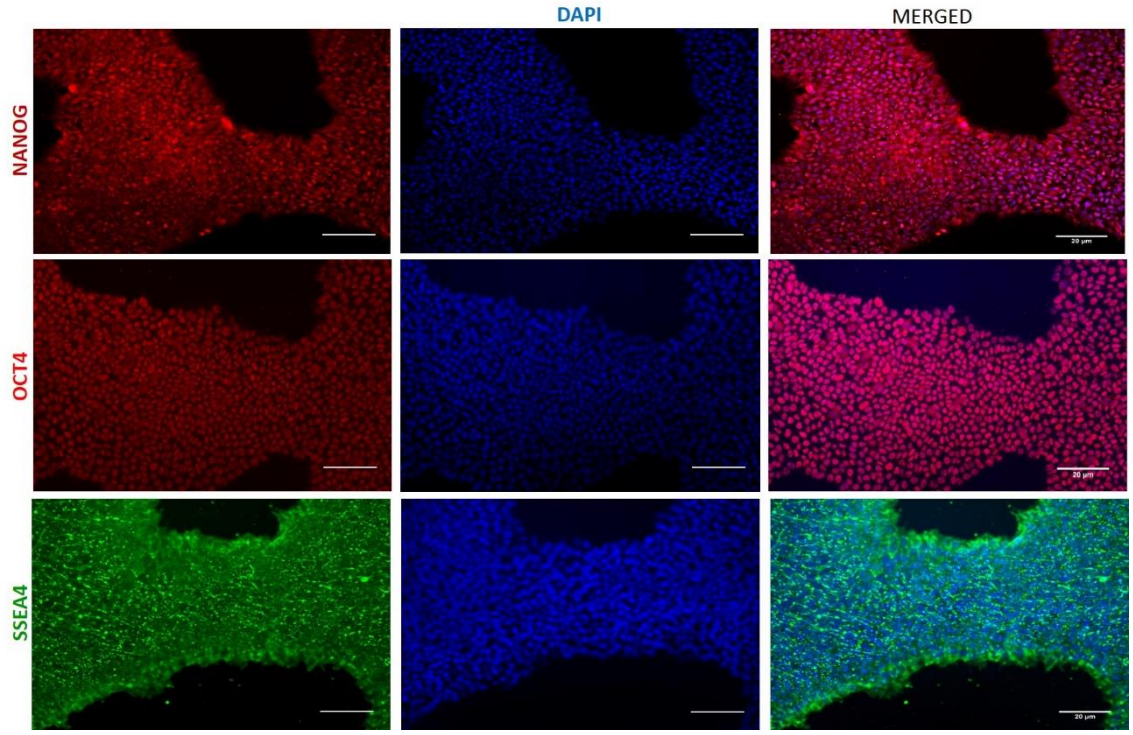

Figure 3.1 – Assessment of the CRISPRi-CCBE1 hiPS cell line pluripotency. Immunofluorescence assay for pluripotency markers shows positive staining for all three markers. The nuclear markers NANOG and OCT4 present clear and strong signal, whereas membrane marker SSEA4 displays weaker signal which could be related to the antibody used not been in the best conditions. Images obtained by widefield microscopy. Scale bars = 20µm.

3.2 *In vitro* differentiation of hiPSCs into Cardiac Fibroblasts

The protocol for hiPSCs differentiation into CFs consisted in a three phases process (Figure 3.2), with each phase being modulated by small molecules to properly conduct the differentiation. The first one is the differentiation of hiPSCs to Cardiac Progenitor Cells (CPCs); the second, is the differentiation of CPCs to proepicardium cells; and finally, the differentiation of epicardium cells to Cardiac Fibroblasts. The differentiation was conducted in monolayer culture since it allows a more controlled specification of the cells and yields a higher purity of the intended cells, unlike the EB method in which the differentiation is mainly spontaneous, despite the addition of molecules to promote the differentiation. Therefore, using the EB method, other cell types are obtained besides the desired cells, resulting in low yields and several different cell types.

To assess the efficacy of the differentiation protocol, we employed multiple molecular techniques, such as qPCR, to verify the genetic profile of cells at different time-points and using several markers specific to each differentiation stage; immunocytochemistry to detect key proteins and further corroborate the qPCR results. Moreover, since the cells

pass through several stages and changes in their morphology during this protocol, for each differentiation stage, we verified and compared our cells with the literature to confirm the success of the differentiation along the protocol.

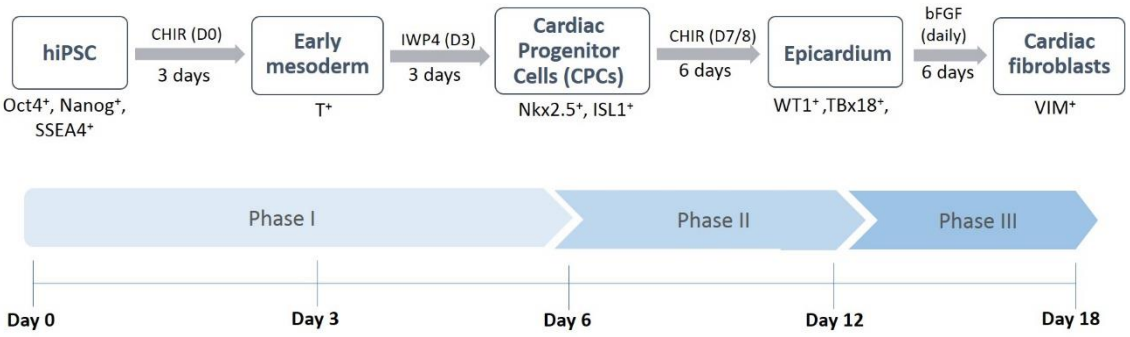

Figure 3.2 – Schematic representation of hiPS cells differentiation into cardiac fibroblasts. Below the boxes are the markers to isolate that specific cell type. Above the arrows are the compounds to induce the differentiation for the next cell type and at which day they are added, while below the arrows is the duration of the step.

3.2.1 Phase I – differentiation of hiPSCs in cardiac progenitor cells

The differentiation of hiPSCs to CPC was an eight days process requiring the use of two small molecules, CHIR99021 (on day 0 of differentiation) and IWP4 (on day 3 of differentiation), to allow the formation of early mesoderm and cardiac progenitor cells, respectively. Both molecules are involved in the modulation of the WNT/ β -catenin pathway, the CHIR molecule acts as an activator of WNT canonical pathway, whereas IWP4 molecule was used as an inhibitor of this specific pathway. The WNT canonical pathway is one of the most essential pathways during the first stage of cardiac commitment. During the embryonic development, the expression of *WNT* has a biphasic role, first, the canonical WNT pathway induces the mesodermal formation while inhibiting the cardiogenesis process, favouring the proliferation and migration of early progenitor cells. Then, according to spatiotemporal context, the WNT/β-catenin canonical pathway is down-regulated by the noncanonical pathway, leading to the initiation of the cardiogenesis process, promoting the differentiation of early mesendoderm progenitor cells towards specific cardiac progenitor lineages.

The CHIR molecule is an inhibitor of the Gsk3 pathway, which in turn inhibits the canonical WNT pathway [103–106]. Therefore, in this way, CHIR functions as an indirect activator of the WNT/ β-catenin canonical pathway that is known to activate the expression of early cardiac commitment markers, such as *BRACHYURY*. In accordance,

the expression of *BRACHYURY* in our differentiation protocol starts two days after the induction with CHIR, being its peak of expression at day 3, the day that marks the early mesoderm stage, as expected (Figure 3.3A).

Contrarily, the IWP4 molecule is an inhibitor of the WNT/ β -catenin canonical pathway, and an activator of the noncanonical WNT pathway[103,104], therefore, when added to the cells, this molecule redirects the *BRACHYURY*$^{+}$ mesendoderm progenitor cells towards a cardiac progenitor fate, marked by the expression of *NKX2*.5 and *ISLET1* genes. This pattern of expression is obtained during our differentiation protocol in which we can observe high levels of expression of both markers at days 5 and 6 of differentiation (Figure 3.3B), confirming the proper differentiation of the hiPS cells into CPCs. Moreover, we can also verify that the *ISLET1* expression begins earlier than *NKX2.5*, starting at day 4 of differentiation. These results were further corroborated by flow cytometry analysis of *NKX2.5* at day 6 of differentiation (Figure 3.3C), the pinpoint of cardiac progenitor stage. According to these results, approximately 86.7% of D6 differentiation cells showed to be NKX2.5 positive (n=3). Interestingly, this result was higher than the percentage obtained by Bao and colleagues[102] that only generated around 55% of *NKX2.5*$^{+}$ cells at D6.

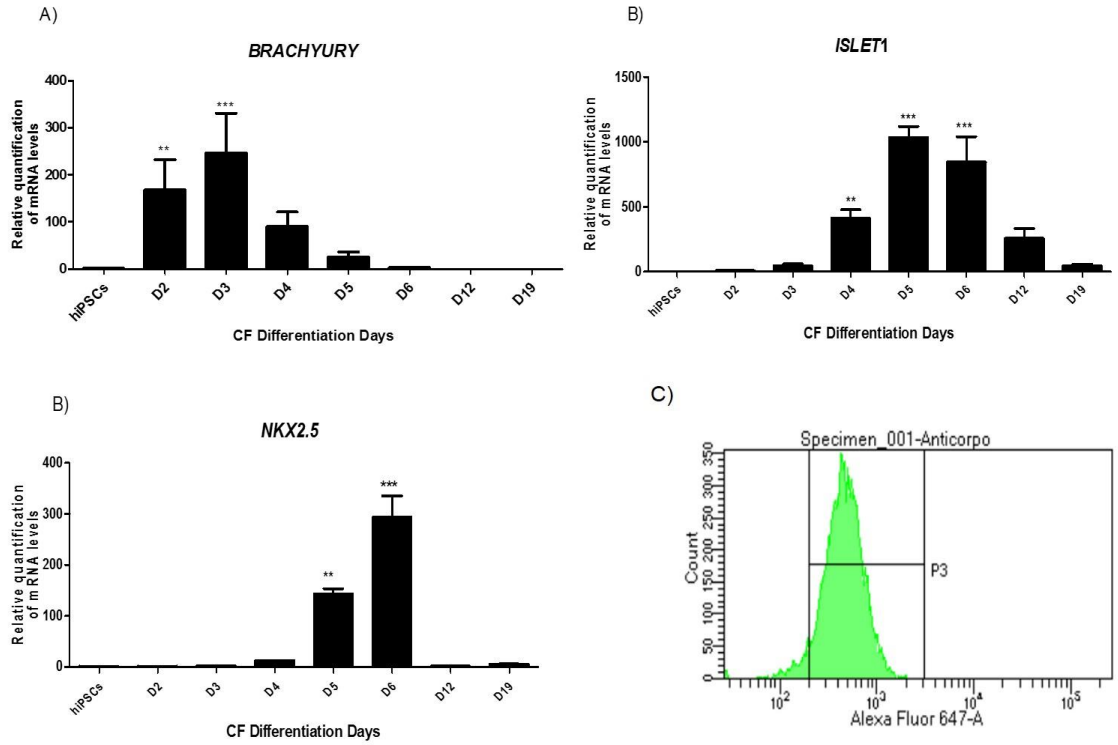

Figure 3.3- Phase I differentiation markers analysis. Relative mRNA expression of A) early mesoderm gene *BRACHYURY* and B) cardiac progenitor genes *ISLET1 a*nd *NKX2.5*. Results are represented as mean± SD of at least two independent biological experiments. One-way ANOVA Newman-Keuls multiple comparison test was performed to compare differences to the control sample. **p<0.01 and ***p<0.001 represent significant results. The samples were normalized to the negative control hiPSCs. C) Representative flow cytometry image for NKX2.5 marker at day 6 of differentiation. Green histogram represents the stained sample and P3 the positive population. Three independent experiments were performed with 10k cells, obtaining the following percentages: 93.1, 82.1 and 84%.

A residual expression of *ISLET1* was also observed at day 12 of differentiation (proepicardial stage), which can be explained by the second heart field origin of these epicardial cells (Figure 3.3B).

Altogether, the qPCR and flow cytometry results strongly indicated a successful differentiation of hiPSCs into cardiac progenitor cells. Moreover, it is also possible to observe morphological changes of these cells during this phase of differentiation (Figure 3.4).

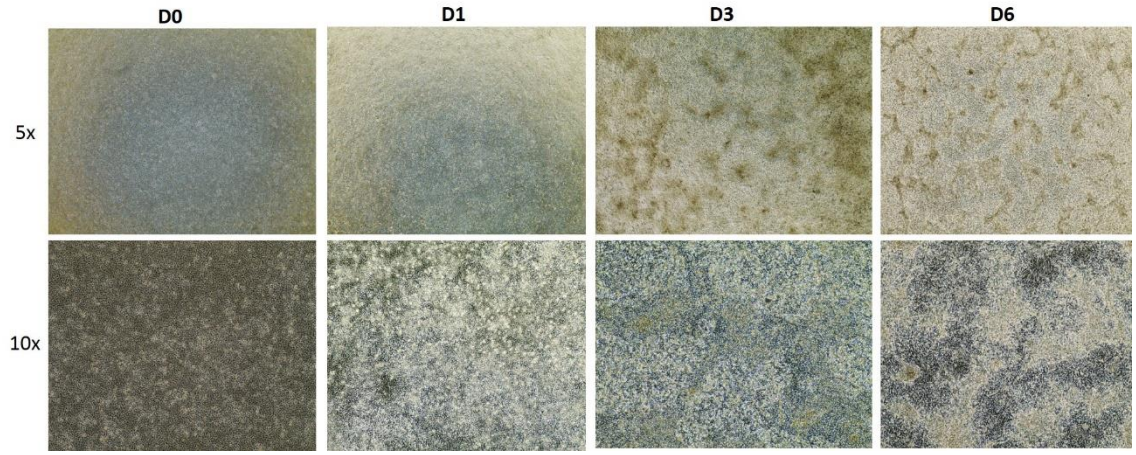

Figure 3.4 – Overview of the cells morphology during phase I of differentiation, resulting in cardiac progenitor cells. During the initial 6 days of differentiation it is possible to observe cells changing from a pluripotent morphology (D0) to an early mesoderm morphology (D3) and finally, to a cardiac progenitor cells morphology (D6). Images were acquired through phase contrast microscope Zeiss XL Core.

3.2.2 Phase II – differentiation of CPCs in proepicardial cells

After successfully achieving the first phase of differentiation and having obtained a high number of CPCs, these cells were further differentiated into proepicardial cells (the second phase of differentiation) for 6 more days. Briefly, at day 6 of differentiation, the CPCs population were re-plated to mechanically select the *NKX2.5*$^{+}$*/ISLET1*$^{+}$ cells, a specific population of second heart field cells that gives rise to proepicardium[37]. After re-plating these cells, on the following two days, we added to the culture medium the CHIR molecule to activate the canonical WNT pathway. This re-activation of WNT pathway induced the proepicardial specification of the cells, characterized by the expression of transcription factors *WT1* and *TBX18*, as we can observe in Figure 3.5A. During heart development, both transcription factors *WT1* and *TBX18* are expressed in the proepicardium and embryonic epicardium and are speculated to help maintain the

epicardial properties of the cells, as well as regulate in the EMT of epicardial derived-cells[107–109].

We also verified a strong upregulation of *TBX5* at day 12 of differentiation. In chicken and mouse heart models, the deletion of this gene causes a myriad of effects, from impaired epicardial-derived cells production and induction of abnormal coronary vasculogenesis, indicating the requirement of *TBX5* for the formation of proepicardium and epicardial cells [110,111].

Furthermore, in Figure 3.5B we can also verify a high expression of *TCF21*, a hallmark gene involved in the epithelial-to-mesenchymal transition of epicardial cells to fibroblasts. The expression of *TCF21* by the proepicardial cells is crucial for the formation of CFs, since it acts as a promoter of epicardial cells EMT and over-time contributes to cardiac fibroblast lineage specification instead of smooth muscle differentiation. Although the mechanisms concerning this lineage specification are still unclear, *Tcf21* null mouse hearts do not form cardiac fibroblasts[18,52], indicating a role for this molecule in this process.

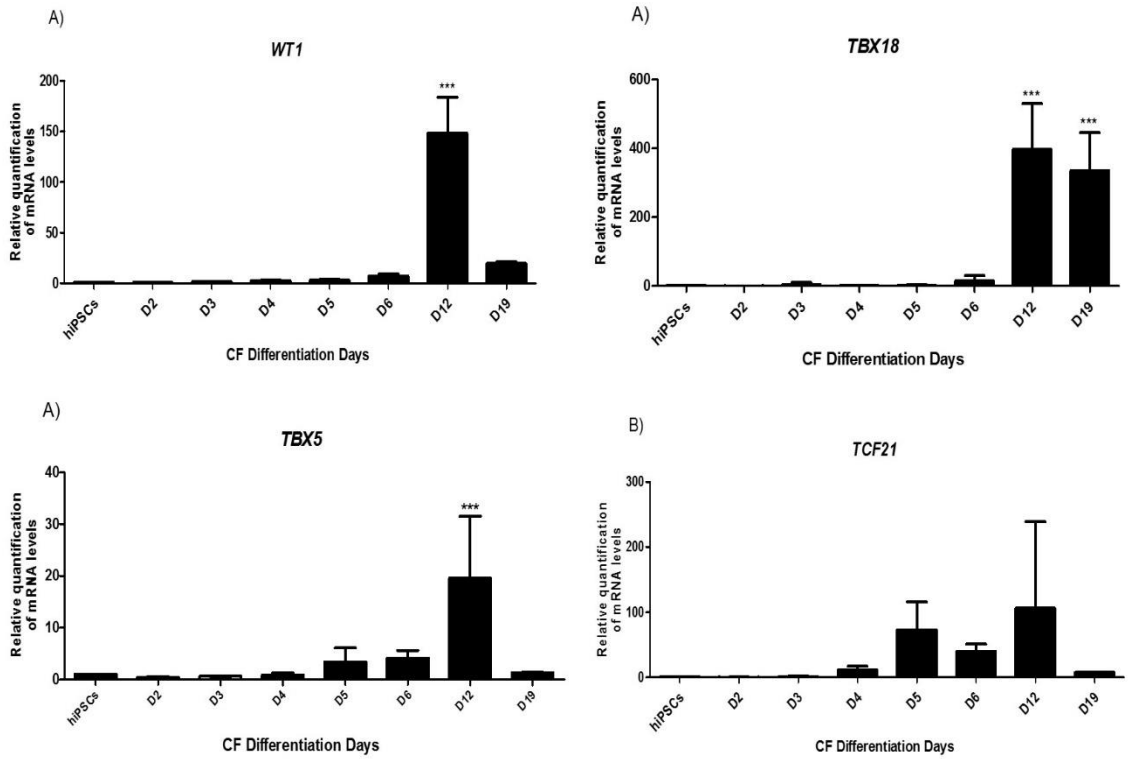

Figure 3.5 - Phase II differentiation markers analysis. Relative mRNA expression of the: A) proepicardium genes *WT1, TBX18* and *TBX5*, B) dual epicardium and cardiac fibroblast marker gene *TCF21*. Results are represented as mean± SD of at least two independent biological experiments. One-way ANOVA Newman-Keuls multiple comparison test was performed to compare differences to the control sample. *** $p<0.001$, represent significant results. The samples were normalized to the negative control hiPSCs.

The proepicardial state of these cells was further corroborated by immunocytochemistry analysis of the WT1 marker at day 12 of differentiation. As we can observe in Figure 3.6, the nucleus of the majority of the cells stained positive for WT1 protein.

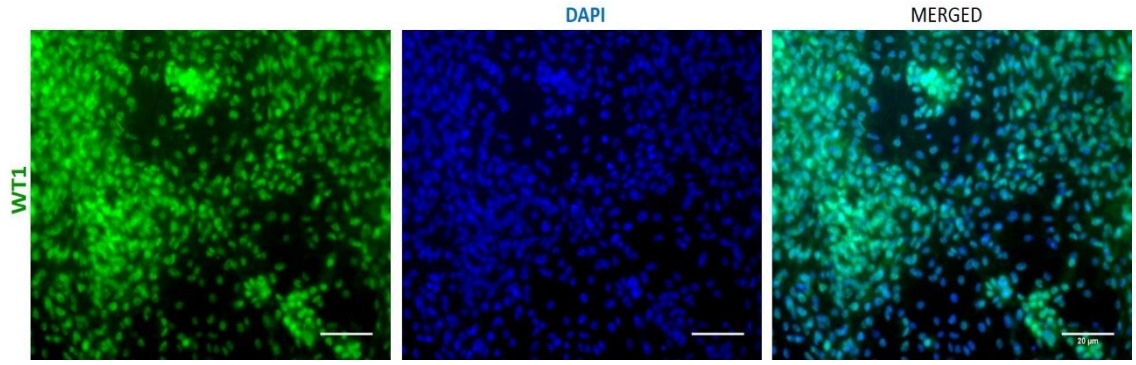

Figure 3.6 – hiPSCs-derived proepicardium immunocytochemistry. Immunostaining for the WT1 proepicardium marker at D12 of differentiation. Images obtained by widefield microscopy and present a strong nuclear signal for WT1 antibody (since WT1 is a transcriptional factor), proving the differentiated cells phenotype. Scale bars = 20µm.

Moreover, the cobblestone morphology of these cells, confirms its proepicardial-like phenotype (Figure 3.7).

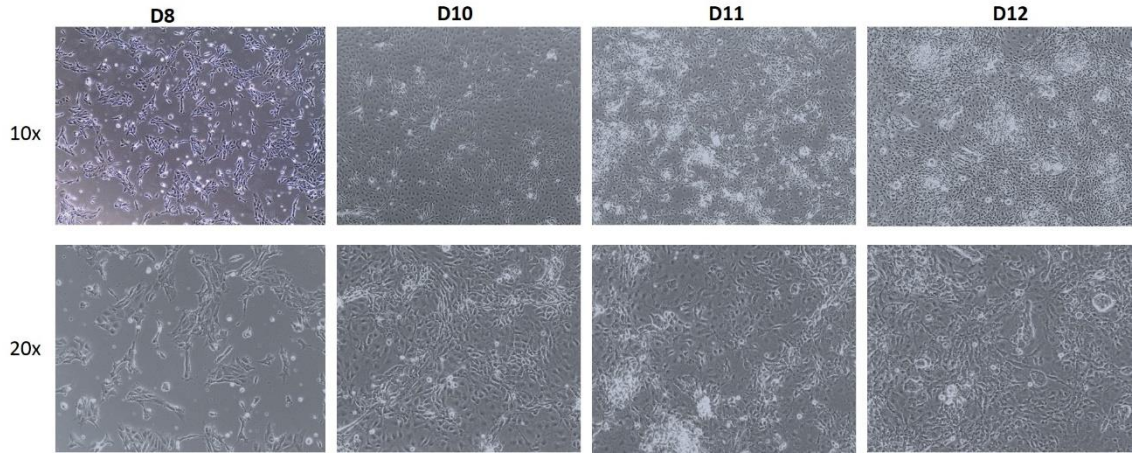

Figure 3.7 – Overview of the cells morphology during phase II of differentiation, resulting in proepicardial cells. After re-plating the cells at day 6 of differentiation, and CHIR supplementation at days 7 and 8 of differentiation, cells differentiate towards an epicardial fate. At day 12 of differentiation, cells present a mature proepicardial phenotype. Images were acquired through phase contrast microscope Zeiss XL Core.

Interestingly, both *TBX5 a*nd *TCF21* were detected at early stages of differentiation (days 5 and 6). The expression of *TBX5* in the cardiac progenitor stage could be explained due to the formation of a small first heart field CPCs population which can express this marker, whereas the expression of TCF21 is a bit surprising. One possible explanation for the early expression of TCF21 is based on the limitations of the *in vitro* differentiation

process, since, despite straining the cells differentiation towards a specific cardiac lineage chemically, some cells may differentiate into other cell types, hence contributing to the expression levels.

3.2.3 Phase III – differentiation of proepicardial cells in cardiac fibroblasts

Similarly to the procedure performed for proepicardial specification of cardiac progenitor cells, the *WT1*$^{+}$*/TBX18*$^{+}$ epicardial cells were further re-plating in order to differentiate those cells into cardiac fibroblasts[37]. To accomplish this, the re-plated cells were submitted to bFGF signalling for six days. The bFGF molecule is long known to be involved in the epicardial cell differentiation as it promotes the EMT, which in turn is responsible for the generation of epicardial derived cells such as CFs[104–106]. At day 19 of differentiation, the protocol was finished, and efficiency assessed by the expression of *TCF21* and *HAND2* genes, and VIMENTIN protein. As observed on Figures 3.5B and 3.8, the cells express *TCF21* and *HAND2* markers, indicating a cardiac fibroblast phenotype.

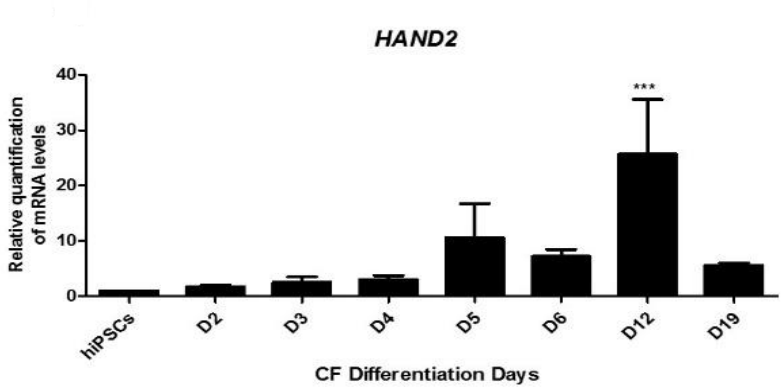

Figure 3.8 - Phase III differentiation markers analysis. Relative mRNA expression of *HAND2* gene. Results are represented as mean± SD of at least two independent biological experiments. One-way ANOVA Newman-Keuls multiple comparison test was performed to compare differences to the control sample. ***p<0.001 represent significant results. The samples were normalized to the negative control hiPSCs.

Moreover, we performed an immunocytochemistry against the VIMENTIN protein, which is usually expressed in the cytoskeleton of cells undergoing EMT, and in this case marking the differentiation of proepicardial cells to CF cells. The result observed in Figure 3.9 shows that the majority of the cells stained positive for VIMENTIN.

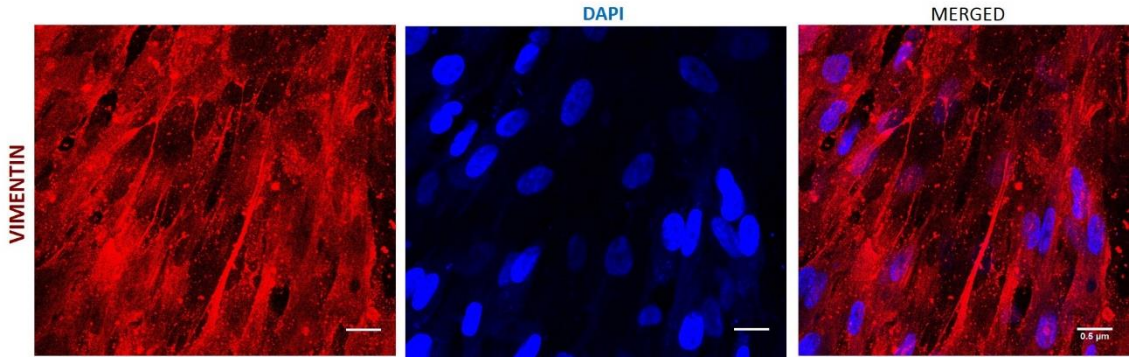

Figure 3.9- hiPSCs-derived cardiac fibroblasts immunocytochemistry. At the end of differentiation (D19) the fibroblasts obtained were re-plated for immunocytochemistry analysis using the VIMENTIN antibody. Images obtained by confocal microscopy. Despite the background signal, it is possible to visualize the vimentin fibres stained, indicating the correct differentiation of proepicardial cells into cardiac fibroblast. Scale bars = 0.5μm.

Considering all the data, and the morphological aspect of the cells (Figure3.10), we were able to differentiate hiPSCs into cardiac fibroblasts cells.

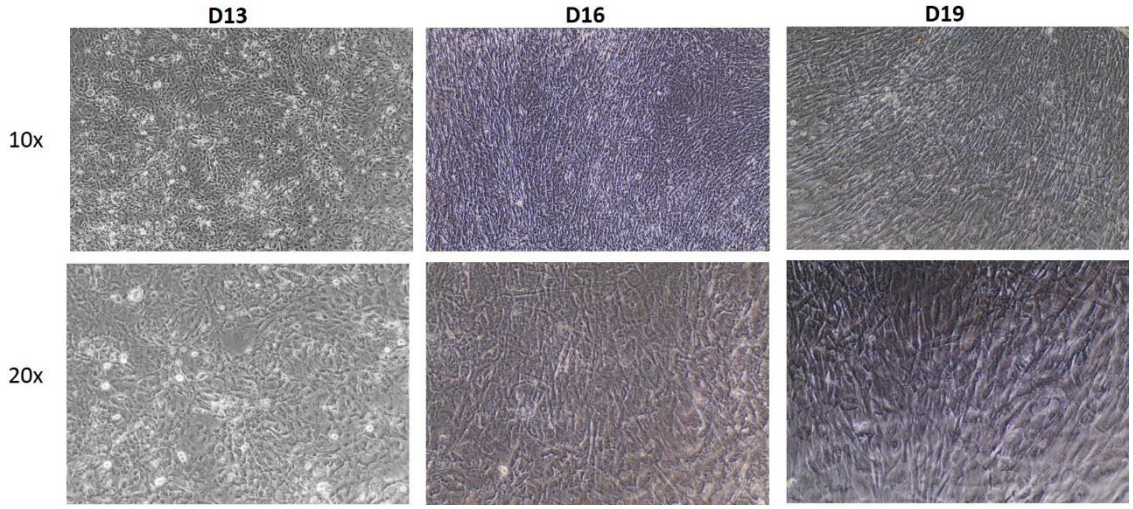

Figure 3.10 – Overview of the cells morphology during phase III of differentiation, resulting in cardiac fibroblasts. At day 13 of differentiation, one day after re-plating proepicardial cells, the cells morphology still resembles proepicardial cells. However, over the course of days due to daily addition of bFGF, cells begin to shape into spindle-like shapes, a typical morphology of fibroblasts. By day 16 of differentiation this morphology is evident and continues to mature until day 19 of differentiation. Images were acquired through phase contrast microscope Zeiss XL Core.

3.3 Modulation of *CCBE1* gene during Cardiac Fibroblasts differentiation

As stated initially, besides producing cardiac fibroblasts we also aimed to verify the expression of *CCBE1* gene throughout the cardiac fibroblast differentiation process, as well as its expression in the obtained CFs.

As it can be observed in Figure 3.11, *CCBE1* presents a peak of expression at day 6 of differentiation – CPC phase – and a basal expression from day 12 to day 19. Previous results from our laboratory, using mice models, presented similar levels of *CCBE1* expression in the embryonic proepicardium to the ones we obtained at day 12 of this differentiation protocol[59,65]. Furthermore, the expression levels of *CCBE1* at day 19 of differentiation are also in concordance with the Affimetrix® results, which hinted a low expression of *CCBE1* in mouse embryonic cardiac fibroblasts.

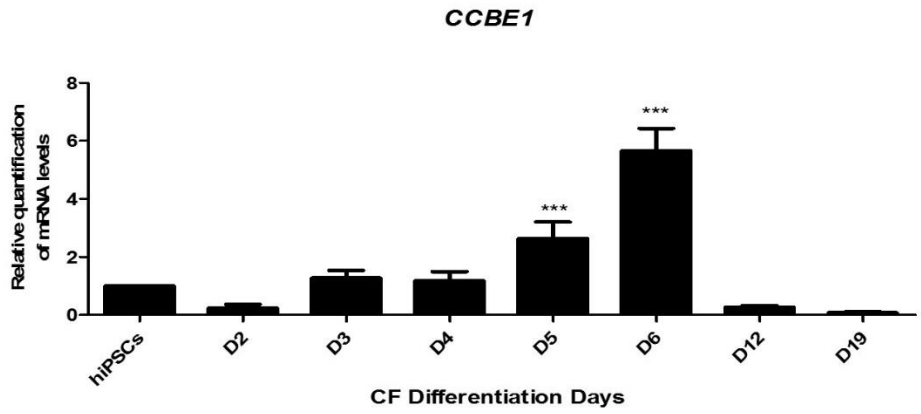

Figure 3.11 – CCBE1 expression during hiPSCs differentiation to cardiac fibroblasts. Relative mRNA expression of *CCBE1* gene at different time points of the differentiation protocol. Results are represented as mean± SD of at least two independent biological experiments. One-way ANOVA Newman-Keuls multiple comparison test was performed to compare differences to the control sample. ***p<0.001 represent significant results. The samples were normalized to the negative control hiPSCs.

After knowing the expression pattern of *CCBE1*, we assessed how the modulation of this gene at its peak of expression, day 6, affects the molecular pathways and genes involved in the formation of human CFs, impacting the yield and/or morphology of these cells. To evaluate this, we firstly determine the acting time of doxycycline (DOX) in undifferentiated hiPSCs. When DOX is added to the CRISPRi-*CCBE1* hiPS cell line, it induces the deactivated Cas9 nuclease fused to the KRAB a repressive domain. This complex with the help of a *CCBE1* specific gRNA will targeting *CCBE1* gene, preventing

its expression. We verified that the addition of DOX to the CRISPRi-*CCBE1* cells for 8 days induced approximately 90% of knockdown (KD) (Figure 3.12).

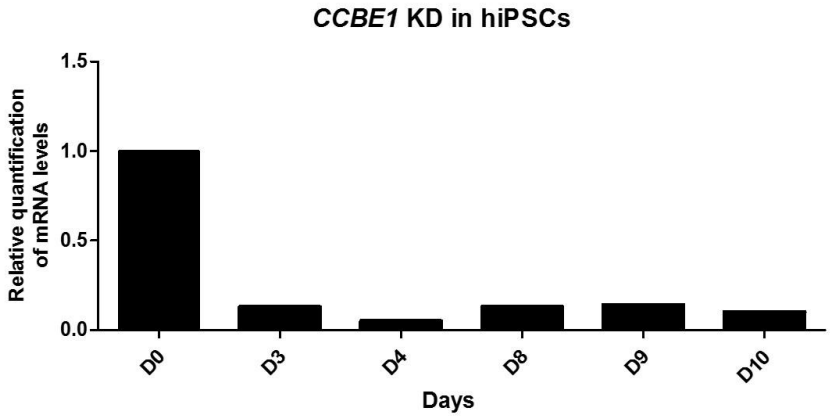

Figure 3.12 - CCBE1 expression during doxycycline knockdown induction in undifferentiated CRISPRi-CCBE1 hiPS cell line. The inhibition of *CCBE1* by doxycycline (DOX) was performed for 8 days. At the 8th day, DOX was withdrawn from cells, therefore days 9 and 10 correspond to the recovery of CCBE1 expression levels. Relative mRNA expression of CCBE1 gene at different time points of the knockdown induction experiment. Results are one independent biological replica. The samples were normalized to the negative control hiPSCs (D0). The knockdown efficiency at D8 was approximately 90%. Data from previous experiments without statistical analysis since only one biological replica was performed.

Taking into account these results, the modulation of *CCBE1* was performed by adding DOX to the CRISPRi-*CCBE1* hiPS cells from day 0 to day 6 of differentiation. As a control of this experiment, we used the CRISPRi-*CCBE1* hiPS cell line without the addition of DOX. Then, we assessed the KD efficiency of *CCBE1* and the expression of several stage-specific cell markers involved in the differentiation process of cardiac fibroblasts, by qPCR analysis.

After 6 days of DOX addition we obtained a 44% KD of *CCBE1* (Figure 3.13). This value differs from the one at pluripotency state, in which we obtained between 60-90% KD of *CCBE1*, probably due to the activation of multiple and simultaneous pathways during a normal differentiation process, leading to a very dynamic environment, in constant changes, therefore hampering the maintenance of the inhibition[97].

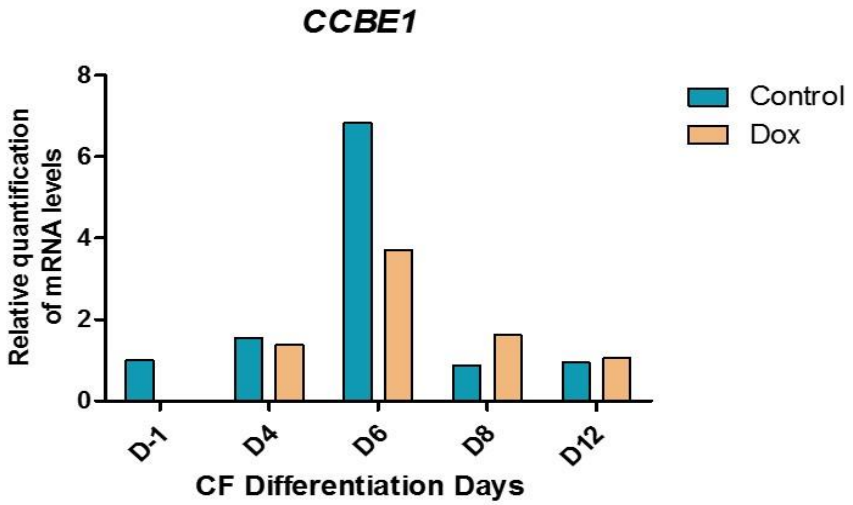

Figure 3.13- Analysis of the CCBE1 levels of expression in the CCBE1 KD experiment, which was performed during the initial 6 days of cardiac fibroblasts differentiation. Analysis of the **r**elative mRNA expression of *CCBE*1 in different time points of the differentiation protocol and in both the conditions: untreated cells (control) and cells treated daily with doxycycline (DOX). All values were normalized to the negative control (without DOX) D-1 of differentiation, the day before starting CCBE1 inhibition. Knockdown efficiency of CCBE1 at D6 of differentiation was 44%. No statistical analysis was performed since this experiment corresponds to only one biologic replica.

Regarding the stage-specific cell markers involved in the differentiation process of cardiac fibroblasts we started by analysing the expression of the mesendoderm marker *BRACHYURY* at day 4 of differentiation. As we can observe in Figure 3.14 the expression levels of *BRACHYURY* were lower in the cells treated with DOX, when compared to the untreated cell. This indicates that *CCBE1* knockdown impairs the differentiation of the stem cells to a mesendoderm fate.

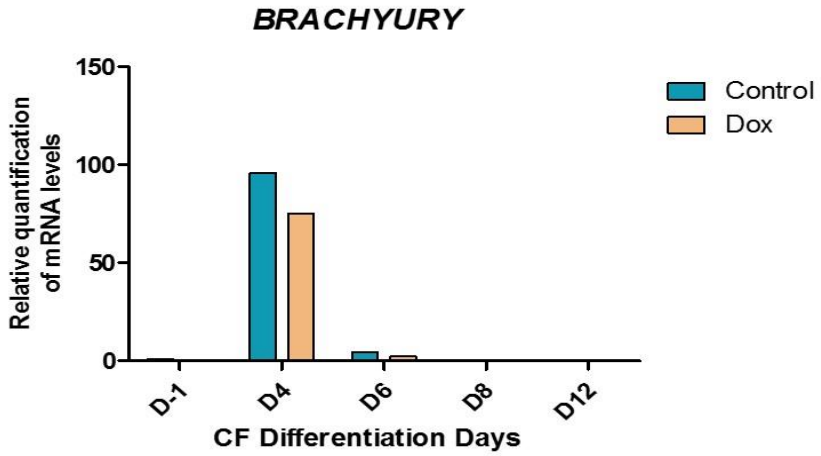

Figure 3.14- Analysis of the Early Mesoderm marker in the CCBE1 inhibition experiment performed during the initial 6 days of cardiac fibroblasts differentiation. Analysis of the **r**elative mRNA expression of *BRACHYURY* in different time points of the differentiation protocol and in both the conditions: untreated cells (control) and cells treated daily with doxycycline (DOX). All values were normalized to the negative control (without DOX) D-1 of differentiation, the day before starting CCBE1 inhibition. No statistical analysis was performed since this experiment corresponds to only one biologic replica.

Next, we verified that the expression of CPC markers, *ISLET1* and *NKX2.5* was also decreased in the cells treated with DOX vs the control condition at day 6 of differentiation Moreover, we also observed a delay in the expression of *ISLET1* in the cells with KD of *CCBE1*. In the normal conditions, *ISLET1* start to be expressed at day 4 of differentiation but in these cells it was just detected at day 6 (Figure 3.15).

Altogether, this might indicate that from day 4 to day 6 the absence of CCBE1 results in a delay of early mesoderm cells to differentiate into cardiac progenitor cells, hinting a role of *CCBE1* (directly or indirectly) in the temporal expression of *ISLET1* and formation of the second heart field progenitor population. These results are in agreement with previous reports from our laboratory, using mouse embryonic *Ccbe1* knockout stem cells, in which the absence of *Ccbe1* affects the formation of progenitor cells, leading to a decreased expression of the early mesoderm marker *Mesp1* and the cardiac progenitor marker *Islet1*. Furthermore, since the differentiation was performed by EBs differentiation technique, it was also possible to observe a smaller size of EBs in the absence of *Ccbe1* expression due to a reduced cell proliferation. Taken together, these results indicate that *Ccbe1* is involved in the promotion of early mesoderm and cardiac progenitor cells formation[63], in both mouse embryonic and human induced stem cells early cardiogenesis processes.

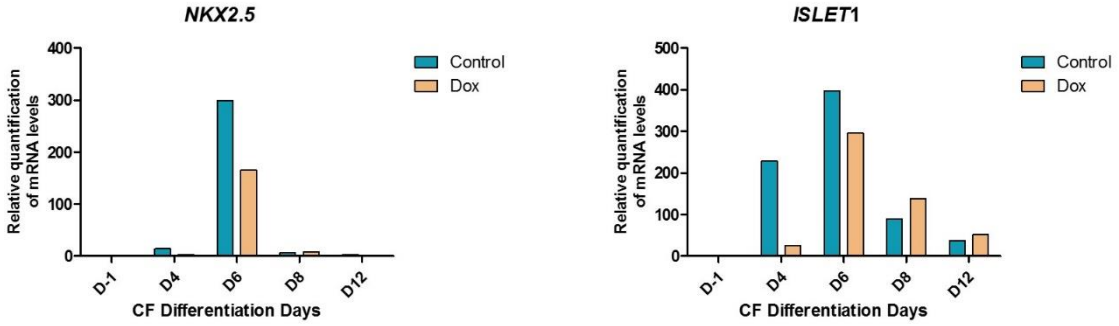

Figure 3.15- Analysis of Cardiac Progenitor markers in the CCBE1 inhibition experiment performed during the initial 6 days of cardiac fibroblasts differentiation. Analysis of the **r**elative mRNA expression of *NKX2.5* and *ISLET1* in different time points of the differentiation protocol and in both the conditions: untreated cells (control) and cells treated daily with doxycycline (DOX). All values were normalized to the negative control (without DOX) D-1 of differentiation, the day before starting CCBE1 inhibition. No statistical analysis was performed since this experiment corresponds to only one biologic replica.

Concerning the expression of proepicardial markers we observed that at day 12 of differentiation, the expression of *TBX18*, *TBX5* and *TCF21* proepicardial markers was higher in the cells treated with DOX comparing to the control cells (Figure 3.16). These

results indicate a role for CCBE1 in the commitment of *ISLET1*$^{+}$*/NKX2.5*$^{+}$ cardiac progenitors towards a proepicardial fate. Curiously, similar expression levels of *WT1* transcription marker were obtained in both treated and untreated conditions, indicating the involvement of several pathways in the proepicardium specification[104–106].

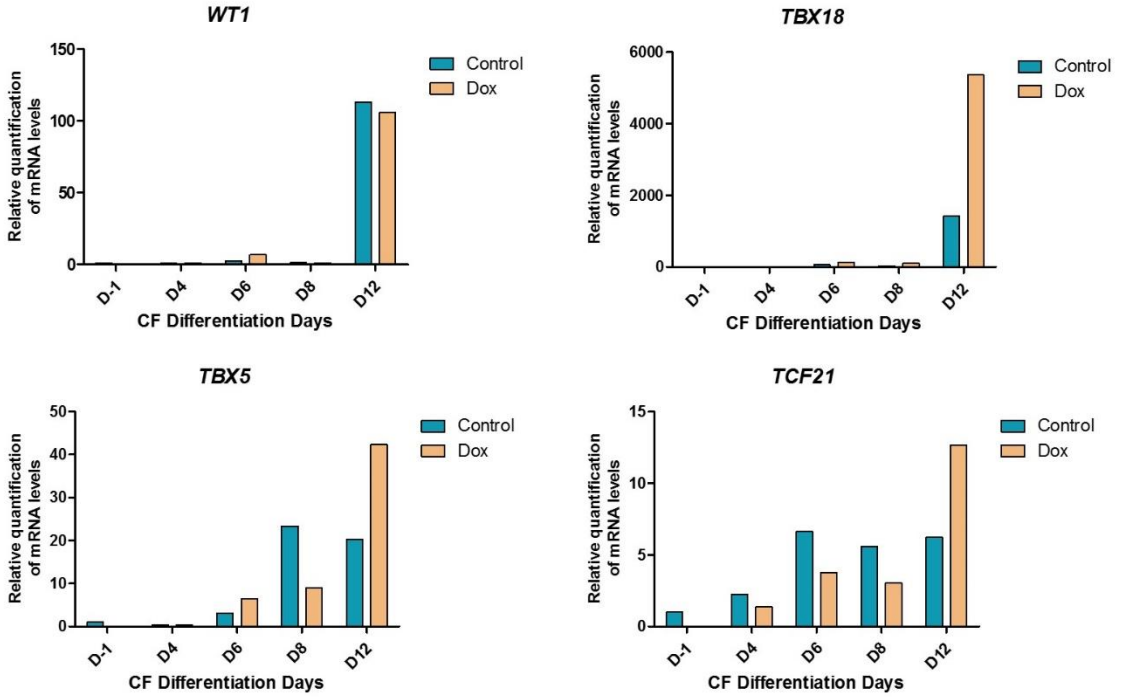

Figure 3.16- Analysis of Proepicardium markers in the CCBE1 inhibition experiment performed during the initial 6 days of cardiac fibroblasts differentiation. Analysis of the **r**elative mRNA expression of *WT1, TBX18, TBX5* and *TCF21* in different time points of the differentiation protocol and in both the conditions: untreated cells (control) and cells treated daily with doxycycline (DOX). All values were normalized to the negative control (without DOX) D-1 of differentiation, the day before starting CCBE1 inhibition. No statistical analysis was performed since this experiment corresponds to only one biologic replica.

Overall, the knockdown of *CCBE1* during the initial stages of CF differentiation negatively affects the expression of cardiac progenitor genes, as already had been observed in mouse cells[63]. More importantly, we observed that by inhibiting *CCBE1*, the expression of proepicardial genes in the end of the proepicardial phase is clearly enhanced, hinting a crucial role of CCBE1 in the regulation of the fate choices between cardiomyocytes and cardiac fibroblasts.

Chapter 4 – Conclusions

Coronary heart disease is one of the most common causes of death worldwide. Moreover, the proportion of individuals suffering from the disease is likely to increase due to an aging global population, thus increasing its prevalence and economic burden. For this reason, it is important to develop novel agents that stimulate angiogenesis to restore blood flow to patients suffering from ischaemic diseases. A possible medical solution to counterattack the loss of CMs and coronary vessels could be the use of 3D pre-vascularized tissue patches containing not only CM, as well as ECs and CFs to promote the angiogenesis in the damaged area and, consequently, increasing the cell viability and migration of CMs towards the injured area allowing its repopulation, and ultimately leading to cardiac regeneration.

With this in mind, our laboratory designed a project to create cardiac tissue patch composed of both hiPSCs-derived ECs and CFs for coronary artery disease therapy purposes. However, in order to create a vascularized patch, it was first necessary to understand the precise mechanisms and interactions occurring between ECs and CFs, responsible for the stimulation of the cardiac angiogenic process. Therefore, one of the main goals was to establish a high yield monolayer culture differentiation protocol to generate cardiac fibroblasts, which in turn could be posteriorly co-cultured with endothelial cells to generate the cardiac patch.

In order to differentiate hiPSCs into CFs we used a recent protocol based on the modulation of the WNT pathway by two small molecules, CHIR and IWP4, that act as an activator and an inhibitor of this pathway, respectively. Using this protocol, we obtained a high amount of cardiac progenitor cells (80-95%), characterized by the expression of *ISLET1* and *NKX2.5*. Moreover, at day 12 of differentiation, when the cells should represent a population of proepicardial cells, we obtained cells expressing not only high levels of the two most important proepicardial/epicardial markers, *WT1* and *TBX18*, as well high levels of the EMT marker *TCF21*, which is expressed in cardiac fibroblast lineage-specific progenitors. The expression of WT1 was also qualitatively assessed through immunocytochemistry analysis which indicated a homogenous expression of this protein in almost all the cells. Lastly, we observed that the obtained CFs expressed the transcription factors TCF21 and HAND2, as well the Vimentin protein. Since *TCF21* and *HAND2* genes are expressed by CFs and VIMENTIN is a specific

protein of CFs, we concluded that we were able to produce CFs from hiPSCs. Nevertheless, a more robust and in dept characterization of the produced CFs should be performed to confirm the quality and functionality of these cells.

Once established the protocol for cardiac fibroblasts differentiation, we assessed the expression levels of *CCBE1* gene throughout the process. Our qPCR analysis showed high levels of *CCBE1* expression during the cardiac progenitor phase of the differentiation protocol. However, at latter stages of the differentiation protocol, the expression of *CCBE1* decreased. Therefore, using a CRISPRi-CCBE1 hiPS cell line, and as a first approach, we decided to modulate this gene expression during the first 6 days of differentiation to assess the impact of an early absence of CCBE1 in the yield and/or morphology of CFs. According to the results obtained, an inhibition of *CCBE1* in the early stage of cardiac differentiation, decreases the expression of CPC markers, but increases the expression of proepicardium markers in the end of the proepicardial phase. These results suggest a crucial role of *CCBE1* in the regulation of the differentiation towards the specification of either a cardiomyocyte or a cardiac fibroblast fate, through proepicardium formation.

As for future work, since *in vitro* differentiated cell types appear to have a more embryonic phenotype when compared with the adult mature cells, it would also be interesting to compare the maturity between hiPSCs-derived CFs and human primary CFs. For that, flow cytometry using a double positive staining for PDGFR-α and VIMENTIN (CF markers), could be performed to compare their relative levels of expression. Furthermore, it would also be interesting to co-culture the derived CFs with pure human umbilical vein endothelial cells (HUVECs, a standard model of ECs) to evaluate the potential contribution of CFs to the angiogenic process. Moreover, co-cultures with pro-immature VEGF-C supplementation, would also allow to ascertain the precise role of *CCBE1* and VEFG-C in the angiogenic process.

Concerning, the *CCBE1* KD experience, some improvements still need to be performed. The most relevant would be the improvement of the KD efficiency at day 6 of differentiation. Instead of only inducing the KD with DOX for six days in the beginning of the differentiation process, the inhibition of *CCBE1* should start earlier before beginning the differentiation protocol (when cells are in a pluripotent state). Thus, the *CCBE1* expression will already be decreased by the beginning the of the differentiation, facilitating the maintenance of its inhibition. Other aspect to improve is the number of

biological replicas which should be at least 3 experiments in the same conditions, as well as technical duplicates to ensure that in the same differentiation there is low well-to-well variation, therefore consolidating the results obtained. Based on the results obtained from this KD experiments it would also be interesting to analyse the expression of all these markers at day 19 of differentiation in the same conditions as this 44% KD experiment.

Overall, we were able to establish a protocol for *in vitro* differentiation of cardiac fibroblasts from hiPSCs and study the role of *CCBE1* in cardiac fibroblasts differentiation. The CFs role in maintaining the homeostatic conditions of the heart and, potentially, inducing cardiac regeneration as an intermediate for angiogenesis have only recently been highlighted. Thus, the establishment of differentiation protocols for this type of cells will allow the study of the molecular pathways, both during the process of CF formation and in the CF *per se*, and their regulation for potential therapeutic approaches. With this in mind, and using a CRISPRi-*CCBE1* hiPS cell line, we performed the modulation of *CCBE1* during the first phase of cardiac fibroblasts differentiation. The preliminary results obtained in this dissertation indicated an involvement of *CCBE1* in the proepicardium formation, regulating the specification of a cardiomyocyte or cardiac fibroblast fate. Therefore, upon future corroboration, this could provide incredible insights on a new regulatory mechanism during the proepicardial differentiation, as well as a potential therapeutic target to promote *in vivo* coronary vessel formation through CCBE1 signalling, and consequently the cardiac tissue regeneration, the main goal of Cardiac Regenerative Medicine.

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